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Molecular detection of *mecA* (or *mecC*) gene in MR-CoNS isolated from infected patients in Hospital Tengku Ampuan Afzan and International Islamic University Malaysia Medical Centre

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Abstract

The emergence of nosocomial infections caused by CoNS has led clinicians and researchers to reconsider the role of CoNS and methicillin-resistant CoNS (MR-CoNS) as important agents of nosocomial infections. The present study was conducted on clinical isolates of MR-CoNS obtained from inpatients in Hospital Tengku Ampuan Afzan (HTAA) and International Islamic University Malaysia Medical Center (IIUM-MC) to detect the presence of *mecA* or *mecA* homologue (*mecC* gene). A total of 40 isolates (33 blood, 4 tissues, and 3 swabs) of MR-CoNS were collected through venepuncture, biopsy, and swabbing techniques respectively, and processed by conventional cultural and biochemical methods, antimicrobial susceptibility tests, and finally confirmation to the species level was done by using conventional PCR assay for known four common clinical species. Methicillin and vancomycin-resistance profile of the isolates was performed by E-test and broth micro-dilution methods. Of the 40 isolates, 38 were identified to be methicillin-resistant (MIC ≥ 0.5µg/mL). The remaining 2 isolates were considered as susceptible to methicillin (MIC ≤ 0.25). All 40 isolates were found to be susceptible to vancomycin, with MIC ranging from 1-4µg/mL. Only *mecA* was identified in the 38 MR-CoNS isolates (95%). The other 2 isolates (5%) that were identified to be methicillin-sensitive by the E-test, also tested negative for the presence of *mecC* gene, thus confirmed to be non-methicillin resistant. The high percentage of *mecA* gene among these MR-CoNS isolates points toward the need for periodic antibiogram surveillance as they are identified to cause difficult to treat infections.

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Introduction

In recent years, an increase in the number of methicillin/oxacillin-resistant coagulase-positive *Staphylococcus aureus* (MR-CoPS) and methicillin/oxacillin-resistant coagulase-negative staphylococci (MR-CoNS) strains have become a serious clinical and epidemiological problem, as resistance to this antibiotic implies resistance to all β-lactam antibiotics and possibly other antibiotics due to the ability to transfer resistance genes such as *mecA* (and its homologue, *mecC*), and other functional genes that are carried in the mobile genetic element known as the staphylococcal cassette chromosome *mec* (SCC*mec*).

Generally, MR-CoPS are more widely reported than MR-CoNS. For MR-CoNS, many studies have been carried out in South East Asia, Europe and North and South America including Mexico (Melendez *et al.*, 2016), Brazil (Botelho *et al.*, 2011), US (Sharma *et al.*, 2001), Sweden (Widerstrom, 2010), London (Xu *et al.*, 2018), Germany (Becker *et al.*, 2004), West Indies (Akpaka *et al.*, 2014), etc. In South East Asia, particularly in Thailand, Seng *et al.* (2017a; 2017b) reported biofilm formation in MR-CoNS and their high prevalence. However, these two reports studied environmental MR-CoNS isolates recovered from various hospital and community/university sites, and not clinical MR-CoNS isolates from patients.

Seng *et al.* (2017a) studied samples collected from the hospital environment such as patients' beds, intravenous poles, surgical and medical wards, medical trolleys, wash-basins, door handles, stethoscopes, nurse stations, the emergency room, the intensive care unit, laboratory clothes, urinals, water taps, and toilets. Their other study (Seng *et al.*, 2017b) studied samples collected from the university environment. This study reported a high prevalence of MR-CoNS from items such as library books, escalators and tables, restroom door handles, wash basin areas, urinary taps and toilets, canteen tables, bank notes and coins used for payment, ATM machines and water dispensers, computer rooms and items such as computer mice, earpieces, keyboards and power buttons, and outdoor surfaces such as handrails, exercise machines, and public buses.

In Malaysia, a study was conducted in Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Bandar Tun Razak, Kuala Lumpur, identified *Staphylococcus epidermidis, S. saprophyticus* and *S. xylosus* from CoNS and MR-CoNS isolates using a multiplex PCR approach with primers specific for each species (Sani *et al.*, 2011). However, the study did not identify the gene(s) responsible for methicillin/oxacillin resistance.

From the above background, it is clear that data concerning molecular characterization of MR-CoNS, particularly detection of *mecA* (or *mecC*) is scarce and to my knowledge, unavailable in Malaysia. For that reason, the present study is designed to fill this existing gap through molecular detection of the *mecA* (or *mecC*) gene in MR-CoNS isolates collected in various wards of Hospital Tengku Ampuan Afzan (HTAA) and International Islamic University Malaysia Medical Centre (IIUMMC).

Therefore, the present study will provide important knowledge on the MR-CoNS species harbouring *mecA* (or *mecC*) gene isolated from infected patients in HTAA and IIUM-MC.

Materials and methods

Sample collection and distribution of MR-CoNS from different clinical samples

A total of 40 isolates of MR-CoNS recovered from blood, swabs, and tissue samples obtained from inpatients aged 3 to 80 were collected from two hospitals (IIUM-MC and HTAA) in a 4-months period from February 2019 to May 2019. All 40 samples were collected through venepuncture, biopsy, and swabbing techniques respectively, and sub-cultured on blood agar media. Out of these 40 isolates, 3 isolates were collected from HTAA and the remaining 37 were collected from IIUM-MC. One isolate per patient was included in the study. All inpatients in the study population were Malay: 26 were males, representing 65% of the collected samples, while the

rest of the cases (14) were females, representing 35% of the collected isolates. The gender distribution is shown in Table 1.

Table 1. Gender distribution of the collected isolates.

Gender	Number	Percentage
Male	26	65%
Female	14	35%

The isolates were obtained from different wards and from inpatients diagnosed with different infectious diseases. The isolates collected from HTAA were obtained from the following wards: Medical, Orthopaedic, Paediatric, Nephrology, Surgical, Cardiology, and Forensic. Meanwhile, isolates collected from IIUM-MC were obtained from the following wards: ICU, Internal Medicine-1, Labour room, Orthopaedic, Special care nursery, Internal Medicine-2, and General surgey-1. The distribution of collected isolates from different wards in the two hospitals is shown in Table 2 and Table 3 below.

Table 2. Distribution of collected samples from different wards of HTAA.

Wards	Number of isolates	Percentage (%)
Surgical	1	9.09
Medical	5	45.45
Orthopaedic	1	9.09
Paediatric	1	9.09
Nephrology	1	9.09
Cardiology	1	9.09
Forensic	1	9.09
Total	11	100

Table 3. Distribution of collected samples from different wards of IIUM-MC.

Wards	Number	Percentage (%)
ICU	15	51.72
Internal Medicine-1	3	10.34
Labour room	1	3.45
Orthopaedic-1	4	13.79
Special care nursery	1	3.45
Internal Medicine-2	2	6.9
General surgey-1	2	6.9
General surgey-2	1	3.45
Total	29	100

More specifically, the distribution of collected isolates according to the type of isolates is shown in Table 4 below.

Table 4. Distribution of collected isolates from both hospitals, IIUM-MC and HTAA.

Samples	Total number of isolates	Number and percentage of isolates in each Hospital			
		IIUM-MC		HTAA	
		No.	%	No.	%
Blood	34	24	60	10	25
Tissues	4	4	10	0	0
Swabs	2	1	2.5	1	2.5
Total	40	29	72.5	11	27.5

The distribution of collected isolates according to diagnosis (of various infectious diseases) is shown in Table 5.

Table 5. Distribution of collected MR-CoNS isolates from inpatients diagnosed with different infectious diseases.

SL	Diagnosis	No. of MR-CoNS isolated	Percentage (100%)
1	Catheter-related blood stream infections	1	2.5
2	Bacteraemia	10	25
3	Sepsis (including 1 urosepsis)	7	15
4	Acute exudative lymphadenitis	1	2.5
5	Infected wounds	4	10
6	Septic shock	4	10
7	Hospital-acquired pneumonia	2	5
8	Cardiogenic shock	1	2.5
9	Chronic obstructive airways disease	1	2.5
10	Spontaneous bacterial peritonitis	1	2.5
11	Eye conjunctiva	1	2.5
12	Recurrent miscarriage with OBS +VE	1	2.5
13	Diabetic Foot Ulcer (DFU)-RAP Amputation	1	2.5
14	UTI	1	2.5
15	Breast carbuncle	1	2.5
16	Necrotizing fasciitis (NF)	1	2.5
17	Post-cardiac arrest for nstemi	1	2.5
18	Acute exacerbation of chronic obstructive pulmonary disease (AECOPD)	1	2.5
19	Total	40	100

Biochemical tests

The isolates were sub-cultured by using nutrient agar media and incubated under aerobic conditions at 37°C for 24 h. Subsequently, morphology observation through subculture and gram stain, catalase test and coagulase test were conducted to re-identify the MR-CoNS. *S.*

aureus subsp. *aureus* ATCC™ BAA-976™ and *Staphylococcus aureus* subsp. *aureus* ATCC® BAA-976™ obtained from IIUM-MC were used as positive control for catalase and coagulase test respectively.

Antimicrobial susceptibility test

Then, all confirmed MR-CoNS isolates were subjected to the Kirby-Bauer disc diffusion method on Mueller Hinton agar (Oxoid, Basingstoke, England). The zones of inhibition were used to classify whether the microorganism was susceptible, intermediately susceptible, or resistant to each antibiotic according to the recommendations in M100-S25 document of the Clinical and Laboratory Standards Institute (CLSI) (CLS, 2018). The nine antibiotics used and their disc contents are shown in Table 6 below.

Table 6. Nine antimicrobial drugs and their disc contents in µg/mL.

SL	Antibiotics	Disc content (µg/mL)
1	Oxacillin[a]	30 µg/mL
2	Linezoid	30 µg/mL
3	Teicoplanin	30 µg/mL
4	Clindamycin	2 µg/mL
5	Erythromycin	15 µg/mL
6	Ciprofloxacin	5 µg/mL
7	Trimethoprim-sulfamethoxazole	1.25/23.75 µg
8	Ceftaroline	30 µg/mL
9	Vancomycin	32 µg/mL

[a] MIC tests were performed to determine the susceptibility of all CoNS isolates to oxacilli.

Molecular characterization of MR-CoNS by conventional PCR assay

DNA Extraction

DNA was extracted from the isolated MR-CoNS using Presto™ Mini gDNA Extraction Kit (Geneaid Biotech Ltd). The identified MR-CoNS were inoculated into 2.0mL of Tryptone Soy Broth/Casein Soya bean digest broth (Oxoid ltd., Basingstoke, Hampshire, England) and incubated at 37°C for 20 h. DNA extraction procedures such as sample preparation, washing, lysis, DNA binding, washing, and elution were performed according to the manufacturer's instructions.

Determination of the Concentration and Purity of DNA

The concentration and purity of the extracted DNA was measured using SimpliNano™ micro-volume spectrophotometer (GE, UK).

Gel electrophoresis

The presence of DNA in all 40 extracted DNA samples was confirmed by the appearance of DNA bands stained with ethidium bromide (EtBr) following 1.5% agarose gel electrophoresis in 1 x TBE buffer.

Primer details

To identify the specific species from each isolate, specific primer pairs for the detection of *rdr* gene (*S. epidermidis*), *sodA* gene (*S. haemolyticus*), *sodA* gene (*S. saprophyticus*), and *nuc* gene (*S. hominis*) were selected on the basis of published nucleotide sequences (Kim *et al.*, 2017; Seng *et al.*, 2017a). To confirm methicillin resistance, *mecA* primer pair was also selected on the basis of published nucleotide sequences (Seng *et al.*, 2017a).

The primers were synthesized by Integrated DNA Technologies, Singapore and delivered in lyophilized form inside a cool ice-box, under -20°C. The specificity of the primers sequences was confirmed by GenBank database using Basic Local Alignment Search Tools (BLAST). The details of the MR-CoNS primers used, including staphylococcal species, target genes, forward primers, reverse primers, primer base pairs (bp), temperature (°C), and references used are listed in Table 3.6 below.

Table 7. Primer details: Staphylococci species, target gene, forward primers, reverse primers, primer base pair (bp), temperature (°C), and references.

Target gene	Primer sequences		Product length	Annealing temperature	References
	Forward	Reverse			
rdr	GGCAAATTTG TGGGTCAAGA	TGGCTAAT GGTTTGTCA CCA	124	65	Kim *et al.*, 2018
sodA	AAACAAACTA TGGAAATCCA TCATG	ATTTGGTAA CATACGTGT TGTG	54	58	Kim *et al.*, 2018
sodA	TGGACACTTA AACCACTCAC TA	CTTCTGATT TGGAGTTA AT	52	55	Kim *et al.*, 2018
nuc	TACAGGGCCA TTTAAAGACG	GTTTCTGGT GTATCAACA CC	177	56.4	Seng *et al.*, 2017a
mecA	TGGCTATCGT GTCACAATCG	CTGGAACTT GTTGAGCA GAG	310	58	Seng *et al.*, 2017a

PCR optimization

Primer dilution

Nine hundred µL of molecular grade water was transferred into a 1.5mL sterile microcentrifuge tube for each primer. To prepare a stock solution of 100 µM, each primer was diluted by adding a specific amount of molecular grade water as stated in the primer specification sheet. The 10 µM required primer working solutions were prepared from the stock by transferring 100 µL of the stock into 1.5mL microcentrifuge tubes containing 900 µL of nuclease-free water. The primer stock and working solutions were kept at - 20°C until time of use. Additionally, primer stock solutions were stored protected from exposure to sunlight.

Sample Preparation

All 40 purified DNA of the isolates were taken from the -80°C freezer were diluted to the required concentrations using values obtained from the SimpliNano™ micro-volume spectrophotometer. The following formula was used:

$M_1V_1 = M_2V_2$.

Where,

M_1 = Required final concentration of DNA (ng/µL)

V_1 = Required final volume of DNA (µL)

M_2 = Initial concentration of the stock DNA (ng/µL)

V_2 = Required volume of stock DNA (µL)

The formula above was used to calculate concentration for each of the 40 samples of purified DNA. V_1 was subtracted from V_2 to obtain the volume of nuclease-free water that should be added to each well of the PCR plate, followed by the forward primers and reverse primers, V_1, and exTEN 2x PCR Master Mix (1st BASE, Singapore) to produce the total reaction mixture volume.

The reaction mixture

The forward and reverse primers (Table 3.6), nuclease free water, each of the 40 diluted samples of extracted DNA, and exTEN 2x PCR Master Mix (1st BASE, Singapore) were added to each of the PCR tubes. The exTEN 2x PCR Master Mix (1st BASE, Singapore) was added last. The final volume of the reaction mixtures in each of the PCR tubes is shown in Table 8 below.

Table 8. Final volumes and concentrations of the PCR reaction mixture.

Component	Volume (µL)	Final concentration
exTEN 2x PCR Master Mix, 200 reactions	12.5	1X
Forward primer, 10 µM	2.5	0.1-1.0 µM
Reverse primer, 10 µM	2.5	0.1-1.0 µM
DNA template	5	< 250 ng
Nuclease free water	2.5	N.A

PCR amplification

The PCR tubes (each containing 25µL final volume) were capped and the contents spinned down briefly. The tubes were placed in the thermal cycler (Eppendorf, New York, USA) with gradient setting and incubated at 95°C to completely denature the template DNA for 2-4 min in the initial denaturation step. 35 cycles of PCR amplification were performed (step 2 to 4 as shown in Table 3.8 were repeated for 35 cycles): subsequent denaturation at 95°C for 30 s, annealing of the complementary primers at 55-72°C for 30 s, and extension at 68-72°C for 1 min. The temperature gradient for annealing was set in accordance with the recommended primer annealing temperatures ranging between 55-65°C (refer to Table 3.9). After the first cycle, the whole process was repeated 35 times. The PCR product extension step was set to 68-72°C for 5-10 min and final extension set to 4°C for 300 s to properly amplify the PCR products. The thermal cycler condition for PCR of the targeted genes is shown in Table 9 below.

Table 9. Gradient reaction and Cycle condition.

SL	Steps	Temperature (°C)	Duration (seconds)
1	Initial denaturation	95	120
2	Subsequent denaturation step	95	30
3	Annealing of the complementary primers for the hybridization step	42-65	30
4	Extension period	68/72	60
5	Final extension	4	300

Quality control

For detection of specific *Staphylococcus* species, *Staphylococcus epidermidis* ATCC® 12228™

reference strain that was obtained from IIUM-MC and nuclease free water were used as positive and negative controls, respectively, as described by Albertsen and Brandt (2018) and Lorenz (2018). For the detection of *mecA* and *mecC*, *S. aureus* ATCC 43300 (*mecA* positive and methicillin resistant) also obtained from IIUM-MC was used as positive control.

Analysis of PCR products

The conventional PCR amplification products were analysed using gel electrophoresis with minor modifications as follows: during loading samples, 50 bp (SMOBIO) or 100 bp (Thermo Scientific) DNA ladder was added to the first well as a molecular weight marker. Then, 2 µL of amplified DNA sample was loaded directly using a pipette into each well of the gel except the first well containing the molecular weight marker.

No loading dye was added to the DNA sample, as the exTEN 2x PCR Master Mix reaction buffer consists of a density reagent and two tracking dyes that act as loading dyes by migrating at the same rate as a 4000 bp and 50 bp DNA fragment in 1% agarose gel. The mould containing the loaded gel was placed into the electrophoresis tank and enough electrophoresis buffer was added to cover the gel to a depth of approximately 1 mm. After that, the gel was run at 80 volts (for small electrophoresis tanks) for 45 min. Then, the gel was taken out of the tank, and placed on the Gel Doc System UV transilluminator chamber (Bio-Rad, California, USA). The gel image was captured, and the DNA fragments were visualized as bands on the gel.

Sequencing of amplicons

Sequence analysis of the PCR amplicons was conducted using Sequence Scanner Software 2, and compared against reference sequences in GenBank using local alignment (BLAST). BLAST is able to detect similar sequences against a given reference sequence in GenBank by using local alignment. Base sequence analysis of the MR-CoNS isolates *sodA* (*S. haemolyticus*), *nuc* (*S. hominis*), and *mecA* genes was performed individually against the GenBank database to obtain the most closely related sequence matches.

Results

Re-identification of the MR-CoNS isolates

After subculture on nutrient agar media and incubation for 24 h, the colonies from all 40 MR-CoNS isolates were unpigmented (whitish in colour), round (cocci), smooth, glistening, slightly convex, butyrous, opaque, and ranging from 3-8 mm in diameter. After gram staining, microscopic examination revealed all 40 isolates to be gram-positive cocci. In the catalase test, rapid and sustained production of gas bubbles indicated a positive test. Few and somewhat sustained production of gas bubbles indicated a weakly positive test. The absence of active bubbling indicated negative catalase test. All 40 isolates produced gas bubbles when treated with hydrogen peroxide. In the tube coagulase test, any degree of clotting that remained in place after tilting the tubes was recorded as positive result. On the contrary, absence of any degree of clotting was recorded as coagulase negative. Generally, all 40 isolates did not show any degree of clotting. Therefore, all 40 isolates were proven to be gram-positive, catalase-positive, and coagulase-negative cocci.

Antimicrobial Susceptibility test

The antibiograms of all the categories of isolates such as blood, tissues, and swabs are shown in Table 10 and Fig. 1 below.

Table 10. Antibiograms of MR-CoNS in all types of clinical isolates: blood, tissue, and swabs.

Antibacterial drugs	Disc contents (µg/mL)	Zone diameter (nearest whole mm)					
		S		I		R	
		No.	%	No.	%	No.	%
Oxacillin[a]	30 µg/mL	-	-	-	-	-	-
Linezoid	30 µg/mL	0	0%	0	0%	40	100%
Teicoplanin	30 µg/mL	1	2.5%	2	5%	37	92.5%
Clindamycin	2 µg/mL	0	0%	1	2.5%	39	97.5%
Erythromycin	15 µg/mL	0	0%	0	0%	40	100%
Ciprofloxacin	5 µg/mL	0	0%	0	0%	40	100%
Trimethoprim-sulfamethoxazole	1.25 /23.75µg	3	7.5%	4	10%	33	82.5%
Ceftaroline	30 µg/mL	0	0%	0	0%	40	100%
Vancomycin[b]	32 µg/mL	-	-	-	-	-	-

See the results of MIC tests against all isolates of MR-CoNS to oxacillin and vancomycin. As per the recommendation given in document M1000-S25 of NCCLS, 2018, MIC tests should be performed to

determine the susceptibilities of all isolates of staphylococci to oxacillin and vancomycin. The disc test does not differentiate between oxacillin -susceptible, -intermediate, and -resistant isolates of CoNS, nor does the test differentiate between vancomycin -susceptible, -intermediate, and -resistant isolates of CoNS.

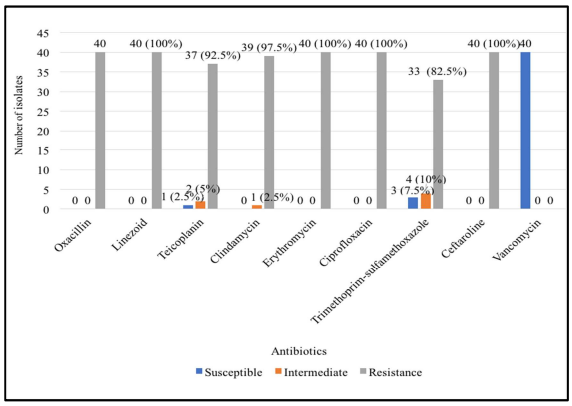

Fig. 1. Antibiograms of MR-CoNS in all categories of isolates: blood, tissue, and swabs. The highest antimicrobial resistance was observed against linezoid, erythromycin, ciprofloxacin, and ceftaroline, while the least resistance was observed against trimethoprim-sulfamethoxazole. The isolates were classified as MDR if they were resistant to more than 3 non-β-lactam antibiotics (Al Laham *et al.*, 2017). The five predominant MDR antibiotic resistance profiles are shown in Table 4.7. MDR profiles of the specific categories of isolates of MR-CoNS are shown in Table 11 below.

Table 11. MDR profiles of all 40 MR-CoNS isolates tested in the present study.

Antibiotic group	Number of isolates
CIP+CPT+CXT+E+OX+VA+LZD+DA+TEC	30
CIP+CPT+E+OX+VA+LZD+DA	1
CIP+CPT+E+OX+VA+LZD+DA+TEC	6
CIP+CPT+CXT+E+OX+VA+LZD+DA	2
CIP+CPT+CXT+E+OX+VA+LZD+TEC	1

Key: CIP= Ciprofloxacin, CPT= Ceftaroline, CTX= Trimethoprim-sulfamethoxazole, E= Erythromycin, OX= Oxacillin, VA= Vancomycin, LZD = Linezoid, DA= Clindamycin, TEC= Teicoplanin.

Oxacillin MIC in MR-CONS

The primary results of oxacillin MIC (30µg/mL) were read at the intersection of the growth-inhibition ellipse with the MIC scale on the strips as recommended in the M. I. C. Evaluator. These preliminary results were further interpreted according to the recommendations given in document M1000-S25 of the CLSI (NCCLS, 2018). Thirty-eight MR-CoNS isolates were oxacillin-resistant, as their MIC values were ≥0.5µg/mL, and the remaining 2 isolates were oxacillin-susceptible, as their MIC values were ≤ 0.25.

Table 12. MDR distribution profiles of specific MR-CoNS isolates tested in this study.

Isolates	No. of Isolates	Distribution of resistance			% MDR
		1 antibiotic	2 antibiotics	≥ 3 antibiotics	
Blood	34	0	0	34	85%
Tissues	4	0	0	4	10%
Swabs	2	0	0	2	5%
Total	40	0	0	40	100%

MIC of Vancomycin in MR-CoNS

The final results showed all 40 MR-CoNS isolates as vancomycin susceptible, with MIC ranging from 1-4 µg/mL.

Genotypic characterization of MR-CoNS

Genetic characterization was conducted to detect the 6 target genes in MR-CoNS species for all 40 MR-CoNS isolates, as described in the following sections.

Quality of Extracted DNA

Each DNA sample showed a single band of intact bacterial chromosomal DNA. The extracted DNA bands and molecular weight marker (100 bp) are shown in Fig. 4.12 below.

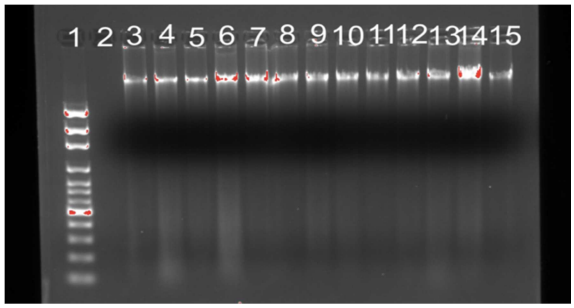

Fig. 2. Agarose gel electrophoresis of extracted MR-CoNS DNA. Lane 1 is the 100 bp DNA ladder; lanes 2-15 are the extracted DNA bands obtained after agarose gel electrophoresis of 13 DNA samples.

The above agarose gel electrophoresis results clearly show the presence and integrity of the extracted DNA from all 40 isolates of MR-CoNS for subsequent use in conventional PCR.

Conventional PCR Assay Results

Conventional PCR Results for Specific Species

Single bands were observed with expected molecular weight markers of 54 bp and 177 bp that signified the presence of the *sodA* gene (*S. haemolyticus*) and *nuc* gene (*S. hominis*), respectively. The DNA size was confirmed using DNA ladders as shown in Fig. 3 and Fig. 4 below.

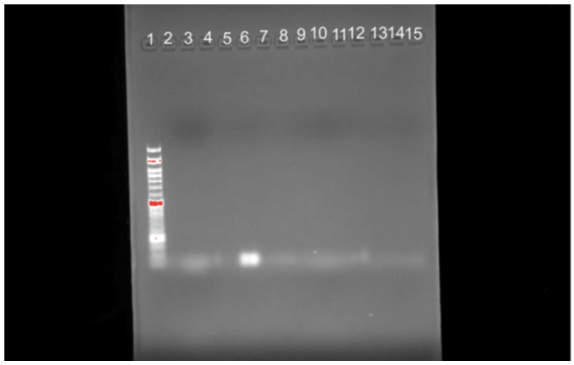

Fig. 3. Agarose gel electrophoresis of *S. haemolyticus* PCR products. Lane 1 is the 54 bp DNA ladder, and lanes 2-15 are single bands of the amplified product obtained after agarose gel electrophoresis.

Fig. 4. Agarose gel electrophoresis of *S. hominis* PCR products. Lane 1 is the 177 bp DNA ladder, and lanes 2-15 show single bands of the amplified product obtained after agarose gel electrophoresis.

The overall prevalence of the bacterial species was as follows: *S. haemolyticus* =13 (32.5%), and *S. hominis* =12 (30%), while *S. epidermidis* and *S. saprophyticus* were not detected. The distribution of bacterial species in clinical samples is shown in Table 4.11, 12 isolates (30%) were *S. haemolyticus* found in blood, and another 12 isolates (30%) were *S. hominis*. For swabs, only one isolate (2.5%) of *S. haemolyticus* was identified. None of the targeted species was identified in tissue isolates.

Table 13. Prevalence of the four targeted CoNS species as measured by conventional PCR.

CoNS Species isolated	Number and percentage of isolates					
	Blood		Tissues		Swabs	
	No.	%	No.	%	No.	%
S. epidermidis	0	0	0	0	0	0
S. haemolyticus	12	30	0	0	1	2.5
S. hominis	12	30	0	0	0	0
S. Saprophyticus	0	0	0	0	0	0
Total	24	60	0	0	1	2.5

Occurrence of mecA (or mecC) Genes in MR-CoNS Species

After 1.5% agarose gel electrophoresis in TAE buffer, all 40 amplified DNA fragments were visualized using the Gel Doc System UV transilluminator chamber. Single bands with expected molecular weight of 310 bp were observed for 38 isolates, while the other 2 isolates did not reveal any bands. Fig. 4.15 below shows the representative bands for the presence of *mecA* genes in 11 isolates.

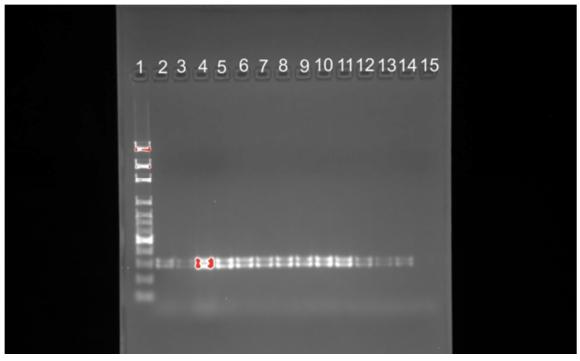

Fig. 5. Agarose gel electrophoresis for *mecA* PCR products. Lane 1 is the 100 bp ladder, lanes 2-12 are MR-CoNS isolates that tested positive for the presence of *mecA* gene, lane 13 is the positive control (*S. aureus* ATCC 43300), and lane 14 is also the positive control (*S. epidermidis* ATCC 12228); lane 15 is the negative control (nuclease-free water).

The bands present after gel electrophoresis signified the presence of *mecA* gene. Out of 40 MR-CoNS

isolates, *mecA* was detected in 38 isolates (95%). Sample-wise, the distribution of isolates tested positive for *mecA* gene in our study was: 32 isolates (84%) from blood, 4 isolates (10%) from tissues, and 3 isolates (7.5%) from swabs. The remaining 2 isolates (5%) did not show any band for the presence of *mecA*. These 2 isolates (5%) that tested negative for the presence of *mecA* were further tested for the presence of the *mecA* homologue, *mecC*, but no bands were observed for the *mecC* gene either. The distribution of *mecA* and *mecC* genes in all three categories of MR-CoNS isolates tested in our study is shown in Table 14 and Fig. 5 below.

Table 14. Distribution of *mecA* and *mecC* in MR-CoNS.

Type of isolates	*mecA*		*mecC*	
	No.	%	N	%
Blood	32	80	0	0
Tissue	4	10	0	0
Swabs	2	5	0	0
Total	38	95	0	0

Sequencing results

Based on the BLAST analysis, all the amplicons displayed 99% to 100% identity with the three corresponding genes *sodA* gene (*S. haemolyticus*), *nuc* gene (*S. hominis*), and *mecA*.

Discussion

Antimicrobial susceptibility test

The distribution of CoNS antibacterial resistance against a panel of commonly used antibacterial drugs in this study is presented in Table 6. One hundred percent (100%) of antibiotic resistance was observed against linezolid, erythromycin, ciprofloxacin, and ceftaroline. The wide use of antibiotics in therapy as well as in prophylaxis has become a major cause for the emergence of resistant bacteria, including MR-CoNS in hospitalized patients. This aggravates the problems already associated with treatment procedures. Comparatively, our findings are quite similar to those reported by Sani *et al.* (2011). Resistance to erythromycin and ciprofloxacin in our study was higher than Sharma *et al.* (2010), (27.9% and 36.3% respectively). Contrasting results were reported by Tayyar *et al.* (2015) and Gilani *et al.* (2012), who found higher susceptibility levels of 100% and very low resistance levels of 2% to linezolid, respectively. In Turkey, Alicem *et al.* (2014) also reported 100% susceptibility to linezolid.

In this study, relatively high resistance levels of 82.5%, 92.5%, and 97.5% were observed against trimethoprim-sulfamethoxazole, teicoplanin, and clindamycin, respectively. These findings were similar to those found by Sani *et al.* (2011), who reported more than 80% resistance to teicoplanin. In contrast, Deyno *et al.* (2018) reported low resistance levels of 50% and 11% to trimethoprim-sulfamethoxazole and clindamycin, respectively. Our results were in contrast to the findings of Tayyar *et al.* (2015), who reported relatively low resistance of 64.1% and 45% to trimethoprim-sulfamethoxazole and clindamycin, respectively. The recommendations given in document M1000-S25 of the CLSI (CLSI, 2018) indicate that it is impossible to differentiate between oxacillin and vancomycin-susceptible, -intermediate, and -resistant isolates of CoNS using the disc diffusion method; further confirmation of methicillin and vancomycin resistance profiles need to be performed by the E-test and broth micro-dilution methods, respectively. Of all the 40 isolates, 38 (95%) were confirmed to be resistant to methicillin, with MIC values ≥0.5µg/mL. The remaining 2 isolates (5%) were susceptible to methicillin, with MIC values ≤ 0.25µg/mL. This MIC value against methicillin is comparatively higher than reported by Sharma *et al.* (2010) (48%), and almost equal to values reported by Paiva *et al.* (2010) (95.4%). The larger sample sizes used in these two studies compared to our study could be a reason behind this slight difference in reported M IC values.

All 40 isolates were identified to be susceptible against vancomycin, as MIC values ranged between 1-4 µg/mL. This was in agreement with many previous studies including Sharma *et al.* (2010) and Paiva *et al.* (2010). However, there have been some emerging cases of vancomycin intermediate-resistance reported in Germany (Soumya *et al.*, 2017), Turkey (Alicem *et al.*, 2013), Italy (Natoli *et al.*, 2009), Australia (D'mello *et al.*, 2008), and the US (Garrett *et al.*, 1999). Jain *et al.* (2013) reported a 15% decrease in

vancomycin susceptibility in 127 CoNS isolates, and Begum *et al.* (2011) reported vancomycin resistance in some *S. haemolyticus* strains. The findings reported by Begum *et al.* (2011) may explain the absence of vancomycin resistance in the predominantly isolated species in our study, *S. haemolyticus* and *S. hominis*, whereby resistance patterns may depend on the strain of the bacteria. Another explanation for the absence of observed vancomycin resistance in our study could be due to the poor reliability of conventional MIC determination methods in CoNS, except for highly resistant species (D'mello *et al.*, 2008). The authors suggest that this is the reason for the emergence of vancomycin-heteroresistant species. This also extends to the E-test, which detects only highly resistant CoNS species, and fails to detect species with lower resistance. Quite revealingly, this is an alarming indication for the future of vancomycin use in the forthcoming months or years.

This study demonstrated the multi-drug resistance profiles of MR-CoNS to more than three antimicrobials; the highest being that to 9 antibiotics. The distribution of multi-drug resistance in this panel of antibiotics is presented in Table 11 and Table 12. Categorically, 34 (85%) blood samples showed multi-drug resistance to more than 3 antibiotics, followed by 4 (10%) tissues samples, and the least being 2 (5%) swab samples. These findings are also in agreement with reports of multiple drug resistance in Thailand by Seng *et al.* (2017), in Germany (Soumya *et al.*, 2017), and in India (Mir & Shikanth, 2013). Meanwhile, Kitti *et al.* (2018) reported high levels of multi-drug resistance against 7-10 antibiotics in MR-CoNS. Recently, a study conducted in the US by Thomas *et al.* (2019) revealed a moderately high level of multi-drug resistance of 73.5% in MR-CoNS. In general, these studies attribute the increase of multi-drug resistance to surgical prophylaxis, exposure to multiple antibiotics, and indiscriminate use of antibiotics.

Conventional PCR assay results

Identification of mecA (or mecC) gene

Conventional PCR assay was run for all 40 isolates to detect the presence of *mecA* and its homologue, *mecC* to confirm methicillin resistance at the molecular level. The distribution of *mecA* and *mecC* genes is presented in Table 14 and Fig. 5. *MecA* was identified in 38 isolates (95%), while the remaining 2 isolates (5%) tested negative for both *mecA* and *mecC*. Sample-wise, 32 isolates (95%) were from blood, 4 isolates (10%) were from tissues, and 2 isolates (5%) were from swabs. The remaining 2 isolates (5%) that were identified by E-test to be methicillin-susceptible and tested negative for the presence of *mecA* gene were further tested for the presence of *mecC* gene. *MecC* gene was not present in 2 isolates, showing that they were non-methicillin resistant. Generally, these results address the limitations of using the disc diffusion method, whereby it is possible to get false positive results for methicillin resistance.

As listed in Table 14, the results are in agreement with previous results in studies from Ethiopia, Brazil, Palestine, Thailand, Mexico, India, London, and Egypt. In the study conducted in Egypt by Rania *et al.* (2017), among 150 CoNS isolates tested for the presence of *mecA* gene and its homologue, *mecC* gene, only 114 isolates tested positive for the presence of *mecA* gene. The remaining 6 isolates that tested negative for the presence of *mecA*, were further tested for the presence of *mecC*, and yielded negative results. This result shows that not all *mecA*-positive staphylococci are resistant to methicillin as tested by the disc diffusion test. This might be due to the minimal expression of PBP2a protein that results in low MIC values (Seng *et al.*, 2017). In a study conducted in Thailand, all 292 isolates of MR-CoNS that were tested for the presence of *mecA* were identified to be positive, but out of the 292 isolates, 15 were not resistant to cefoxitin, a surrogate for oxacillin.

Identification of common species of MR-CoNS harbouring mecA (or mecC) genes

To identify the targeted species in our study, conventional PCR assay was run for all 40 isolates. The distribution of species is presented in Table 13 and Fig. 4. Out of 40 collected isolates, *S. haemolyticus* was the most commonly detected species (13/40, 32.5%), followed by *S. hominis* (12/40, 30%). However, neither *S. epidermidis* nor *S. saprophyticus* were identified in

the isolates. In blood samples, 12 isolates (30%) were *S. haemolyticus* and all 12 isolates (30%) were *S. hominis*. For swabs, only 1 *S. haemolyticus* isolate (2.5%) was identified. In tissue samples, none of the targeted organisms were identified.

In HTAA, the isolates that tested positive for *S. haemolyticus* were collected from the following wards: medical (1/40, 2.5%), surgical (3/40, 7.5%), and orthopaedics-1 (1/40, 2.5%). Isolates that tested positive for *S. hominis* were collected from the medical (3/40, 7.5%) and orthopaedics (1/40, 2.5%) wards. In IIUM-MC, the isolates that tested positive for *S. haemolyticus* were collected from the ICU (5/40, 12.5%), orthopaedics-1 (2/40, 5%), and internal medicine-1 (1/40, 2.5%). Isolates that tested positive for *S. hominis* were collected from the ICU (5/40, 12.5%), general surgery-1 (1/40, 2.5%), general surgery-2 (1/40, 2.5%), and special care nursery (1/40, 2.5%).

Table 15. Number of patients with methicillin resistance and their diagnosis in different wards of HTAA.

SL	Wards	Underlying diseases	No. of inpatients
1	Surgical	Right breast carbuncle	1
2	Orthopaedic	Infected wound	2
3	Medical	Diabetic Foot Ulcer (DFU)-RAP Amputation	1
		Peritonitis	1
		Septicemia	2
		Septic shock secondary to acquired pneumonia	2
		Bacteremia	3
		Sepsis	2

Table 16. Number of patients and their diagnosis in different wards of IIUM-MC.

SL	Wards	Underlying diseases	No. of inpatients
1	ICU	Multiple infections of wounds	2
		Cardiogenic shock	2
		Septic shock	3
		Bacteremia	7
		LL Necrotising fasciitis	1
2	Orthopaedic-1	Septicemia	5
3	Internal medicine-1	Post cardiac arrest for nstemi	1
4	General Surgery-1	Spontaneous Peritonitis and sepsis	2
5	Special care nursery	Sepsis and eye conjunctiva	3

Hence, in our study, most *S. hominis* isolates were collected from the ICU wards (30%), and were obtained from one hospital, IIUM-MC. Generally, isolates from both hospitals were obtained from inpatients diagnosed with infections as summarized in Table 5.1 and Table 5.2 below.

These findings are in agreement with previous studies reported by Tayyar *et al.* (2015), Seng *et al.* (2017a) and Ternes *et al.* (2013). Seng *et al.* (2017a) reported that 251 MR-CoNS isolates were collected from the following wards: medical (84.3%), ICU (75.7%), and surgical (71.4%). Tayyar *et al.* (2015) reported that out of 223 CoNS isolates collected from 7 wards, the highest number of CoNS isolates (92, 41.2%) was collected from ICU. Among these findings, similar cases of underlying diseases were also diagnosed. In this study, most isolates (82.5%) were sampled from blood (Table 14) of inpatients diagnosed with bacteremia (Table 16 and Table 17). Similarly, Tayyar *et al.* (2015) in his study reported that among 223 CoNS isolates collected from 7 wards, 69 (30.9%) were isolated from blood of patients diagnosed with bacteremia.

In many previous studies (Sharma *et al.*, 2010; Soumya *et al.*, 2017; Gilani *et al.*, 2016; Tayyer *et al.*, 2015; Nahaei *et al.*, 2015; Sani *et al.*, 2011 and others), *S. epidermidis* was the most commonly isolated species among MR- CoNS isolates, followed by *S. haemolyticus,* then *S. hominis*. Although several studies identified *S. epidermidis* as the predominantly isolated MR-CoNS species, few studies have identified *S. haemolyticus* as the most commonly isolated MR-CoNS species.

The species distribution of MR-CoNS in our study conforms to previous reports. For example, in a study conducted in Thailand by Kitti *et al.* (2018), 18 of 31 isolates were *S. haemolyticus* (58%), followed by 3 *S. epidermidis* isolates (9.7%) and the least being 1 *S. hominis* isolate (3.23%). However, although *S. haemolyticus* prevalence was similar to our study, the prevalence of *S. hominis* varied largely from our study. In our study, *S. hominis* was the second most-commonly isolated species (12/40, 30%), but Kitti *et al.* (2018) reported *S. hominis* as the least isolated

species (1/40, 3.23%). Similarly, in another study also conducted in Thailand by Seng *et al.* (2017), among 251 MR-CoNS isolates, *S. haemolyticus* was the most commonly recovered isolate, with a prevalence of 41.1%, followed by *S. epidermidis* with a prevalence of 30.1%. In another study conducted in Nigeria by Azih *et al.* (2013) *S. haemolyticus* was identified as the most commonly isolated MR-CoNS species, with a prevalence of 28.3%. However, in contrast to our study, this study did not report recovery of *S. hominis*. Finally, similar to our study, the majority of isolates collected by Azih *et al.* (2013), Seng *et al.* (2017) and Begum *et al.* (2011) were isolated from blood samples collected from ICU wards.

However, the absence of *S. epidermidis* in our study may be explained by the prevalence of *S. epidermidis* in isolates from patients with indwelling medical devices such as orthopaedic prostheses, valvular prostheses, central and peripheral venous catheters, pace-makers, and artificial heart valves (Seng *et al.*, 2017; Garcia *et al.*, 2004; Begum *et al.*, 2011). Our study reports only one *S. epidermidis* isolate from one patient with a catheter-related blood stream infection. The presence of *S. saprophyticus* has been linked to urinary tract infections in females (Becker *et al.*, 2014; Sarathbabu *et al.*, 2013; Cunha *et al.*, 2004). Likewise, in our study, one isolate was recovered from a urinary tract infected patient. Generally, low prevalence of *S. epidermidis* and *S. saprophyticus* have been reported in previous studies. Besides the type of clinical samples used in our study, our sample size of 40 compared to the sample size used in the aforementioned studies may also be the reason for failure to recover *S. epidermidis* and *S. saprophyticus* from our isolates.

Conclusion

The issue of antimicrobial resistance in bacteria from clinical isolates such as *Staphylococcus* species is very serious and of high relevance to modern medicine and to treatment strategies in particular. Our study investigated antibiogram and molecular characterization of 40 MR-CoNS isolates collected from 15 wards of two hospitals: HTAA (7 wards) and IIUM-MC (8 wards). One hundred percent (100%) of antibiotic resistance was observed against linezolid, erythromycin, ciprofloxacin, and ceftaroline.

Followed by resistance levels of 82.5%, 92.5%, and 97.5% against trimethoprim-sulfamethoxazole, teicoplanin, and clindamycin, respectively. Generally, all 40 MR-CoNS isolates were found to be resistant to three or more antibiotics. Therefore, our results show that MR-CoNS was present in various wards of HTAA and IIUM-MC, especially in the medical, surgical, and orthopaedic wards of HTAA, and the ICU, orthopaedic-1, internal medicine-1, general surgery-1, and special care nursery of IIUM-MC.

Four types of staphylococcal species that were investigated from 3 categories of MR-CoNS isolates (blood, tissues, and swabs) were *S. epidermidis, S. haemolyticus, S. saprophyticus,* as well as *S. hominis*. Unsurprisingly, not all of these species could be recovered from the isolates. Out of 40 MR-CoNS isolates, only two were successfully recovered: *S. haemolyticus* and *S. hominis*.

MecA was identified in 38 isolates (95%), while the remaining 2 isolates (5%) tested negative for both *mecA* and *mecC*.

Recommendations

This study shows that several antibiotics are no longer effective in the treatment of MR-CoNS infections, hence, more clinical and laboratory-based studies are needed to enable all stakeholders to be aware of the mechanisms involved in the development of antibiotic resistance in CoNS. These further studies may include the search for other genes apart from *mecA* and its homologue *mecC* that may also be responsible for antibiotic resistance, and molecular typing of isolates, especially by using pulsed-field gel electrophoresis, multilocus sequencing or full genome sequencing. In addition to that, because *mecC* was not identified in any of the MR-CoNS isolates in our study, further studies with larger sample size and more diverse clinical samples are highly needed.

A new generation of antibiotics and novel treatment schemes that focus on combating antibiotic resistance

in CoNS against methicillin and to other antibiotics should be monitored with higher vigilance in order to better tackle the problem of increasing multi-antibiotic resistance in MR-CoNS.

Specifically, because there are few studies that have already reported vancomycin intermediate-resistance and vancomycin-resistance in CoNS, further research, screening and surveillance should be conducted regularly on vancomycin resistance profiles in CoNS. This will help physicians to be more confident with their current prescriptions for combating resistant species of CoNS. Last but not least, in order to combat the increasing burden of multi-drug resistance as observed in our study and reported in previous studies, healthcare providers should adhere to both preventive and control measures against the horizontal spread of resistance.

Ethical approval

Approvals to carry out research and collect samples from both HTAA and IIUM MC Microbiology laboratories were obtained from the appropriate committees and respective authorities. These committees and authorities are listed below:

1. National Medical Research Register (NMRR)
2. Medical Research & Ethics Committee (MREC)
3. IIUM Research Ethics Committee (IREC)
4. Kulliyyah of Medicine Research Committee (KRC)
5. Kulliyyah of Medicine Postgraduate Committee (KPGC).

Conflicts of interest

The author declare no conflict of interest.

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