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Antimicrobial resistance of methicillin-resistant coagulase negative staphylococcal species in two government hospitals in Kuantan, Malaysia

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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. This 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 determine their antimicrobial resistance profile. The isolates were cultured from clinical samples of blood, tissues, and swabs. Forty 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, biochemical methods and antimicrobial susceptibility tests. 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 ≥ 0.5 µg/mL). The remaining 2 isolates were considered as susceptible to methicillin (MIC ≤ 0.25). All 40 isolates were found to be susceptible to vancomycin (MIC=1-4 µg/mL). All 40 isolates were also tested for phenotypic antimicrobial susceptibility profile using the Kirby and Bauer disc diffusion method. Resistance rates to linezolid, erythromycin, ciprofloxacin, and ceftaroline were found to be 100%. Resistance rates to trimethoprim-sulfamethoxazole, teicoplanin, and clindamycin were found to be 82.5%, 92.5%, and 97.5% respectively. Thus, all the isolates revealed multi-drug resistance profiles to more than 3 antimicrobials; the highest being resistance to 9 antibiotics, except one isolate from swab. This points toward the need for periodic antibiogram surveillance as they are identified to cause difficult to treat infections.

Contribution/Originality: This is the first study to detect MR-CoNS from the clinical isolates in various wards of HTAA and IIUM-MC, particularly in the medical, surgical, and orthopaedic wards of HTAA, and in the ICU, orthopaedic-1, internal medicine-1, general surgery-1, and special care nursery of IIUM-MC.

1. 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 [1] Brazil [2] US [3] Sweden [4] London [5] Germany [6] West Indies [7] etc. In South East Asia, particularly in Thailand, reported biofilm formation in MR-CoNS and their high prevalence [8]. 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. [9] 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 [9] 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, Automated Teller Machine (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 that 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 [10]. However, the study did not identify the gene (s) responsible for methicillin/oxacillin resistance. From the above background, it is clear that data concerning antimicrobial resistance profile of MR-CoNS is scarce, and to our knowledge, unavailable in Malaysia. For this reason, the present study is designed to fill this existing gap by determining the antibiogram in MR-CoNS isolates collected in various wards of HTAA and IIUM-MC. Therefore, the present study will provide important information about the antibiogram of MR-CoNS against antibiotics commonly used for their treatment in HTAA and IIUM-MC.

2. METHODOLOGY

2.1. Description of the Study Area

MR-CoNS isolates were collected from Tengku Ampuan Afzan Hospital (HTAA) and International Islamic University Malaysia Medical Centre (IIUM-MC), Kuantan. HTAA is the largest government hospital in Pahang (State General Hospital) located in Kuantan. HTAA is also a teaching hospital for IIUM medical students in addition to the IIUM Medical Centre. Re-identification of isolates and conventional PCR assay was conducted at the Microbiology Research Laboratory, Basic Medical Sciences (BMS) Department, Kulliyyah of Medicine, IIUM.

2.2. Study Design

This was a laboratory-based, cross-sectional descriptive study on clinical isolates of MR-CoNS.

2.3. Study Population

MR-CoNS was isolated from inpatients and outpatients admitted/attending HTAA, Kuantan.

2.4. Inclusion Criteria

All methicillin-resistant CoNS species isolated from clinical samples obtained from infected patients in HTAA by the Pathology Department/Microbiology section were included in the study.

2.5. Exclusion Criteria

Methicillin-susceptible CoNS isolated from clinical samples obtained from infected patients in HTAA and IIUM Medical Centre (IIUM MC) by the Pathology Department/Microbiology section were excluded from the study.

2.6. Sample Size Determination

Due to time and funding limitations, single proportion formula for calculating expected precision of the estimate for the right sample size was used to calculate a convenient sample size of 40 isolates of MR-CoNS for the present study by using the average prevalence of each of the 4 MR-CoNS isolates collected in recent studies [11]. This formula is shown below:

$d = z \times \sqrt{p} \times (1 - p) / \sqrt{n}$,

Where,

z = 1.96 for a confidence level (α) of 95%.

p = Proportion (expressed as a decimal).

n = Sample size.

d = Expected precision of the estimate.

Table 1. Average prevalence of each of the 4 MR-CoNS isolates collected in recent studies.

Species	Average prevalence (%)	Recent publications
S. epidermidis	60.-85	Miragaia, et al. [12]
S. saprophyticus	9-12	Xu, et al. [5] and Al Laham [13]
S. haemolyticus	41.1-93.2	Seng, et al. [8] and Martínez-Meléndez, et al. [1]
S. hominis	1.4-30	Seng, et al. [8]; Botelho, et al. [2] and Sani, et al. [10]

The above Table 1 prevalence values were substituted in the above formula as shown below:

S. epidermidis: $d = 1.96 \times \sqrt{0.725} \times (1 - 0.725) / \sqrt{40} = \pm 13.838\%$

S. saprophyticus: $d = 1.96 \times \sqrt{0.105} \times (1 - 0.105) / \sqrt{40} = \pm 9.5\%$

S. haemolyticus: $d=1.96 \times \sqrt{0.67150000000000001} \times (1 - 0.67150000000000001) / \sqrt{40} = \pm 14.555\%$

S. hominis: $d = 1.96 \times \sqrt{0.157} \times (1 - 0.157) / \sqrt{40} = \pm 11.274\%$

2.7. 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).

2.8. Sampling Method and Sample Collection

Isolated MR-CoNS were sampled via convenience sampling method. A total of 40 isolates were collected from the Microbiology Laboratory, Pathology Department at HTAA and IIUM-MC over a period of 3 months (from the 30th of January to 30th of April in 2019). The isolates were collected from various types of patient specimens depending on type of infection, including blood, tissues, and swabs.

2.9. Patient's Demographic Data

Demographic data (patient's age, sex, race, type of specimens, underlying disease (neoplasm), episode of MR-CoNS infections, types of intra-vascular device and diagnosis) were obtained from hospital case files. The data was

recorded on an excel worksheet, which was then exported to SPSS for analysis. All patient data was kept strictly anonymous. The SPSS results were presented as frequency tables.

2.10. Sub-Culturing

The isolates were taken from the -80°C freezer and suspended in a water bath for approximately one hour to thaw. Inoculating loop, 70% alcohol, permanent marker, Bunsen burner, matches, and 70% alcohol for sterilization of materials were prepared; the bench was wiped using 70% alcohol to maintain an aseptic environment. The nutrient agar plates were taken out of the 4°C fridge and placed in an incubator for an hour in order to dry any moisture present on the agar surface. The preserved isolates were taken out of the -80°C freezer and placed in a water bath for an hour to thaw. The plates were labelled accordingly with date of sub-culturing and code number of the isolate. A sterile wire loop was used to take a loop full of colonies from the preserved isolates, and then streaked four times onto the surface of the nutrient agar under aseptic condition to obtain single isolated colonies. The plates were incubated under aerobic conditions at 37°C for 24 h. After 24 h, the plates were removed from the incubator and examined.

2.11. Gram Staining

2.11.1. Smear Preparation

2.11.1.1. Procedures

The purpose of preparing a smear is to fix the bacteria onto a slide and to prevent the sample from being lost during staining procedures. To accomplish this, the following procedures were performed:

Grease pencil, inoculating loop, 70% alcohol, clean microscope slides, permanent marker, Bunsen burner, matches, and 70% alcohol for sterilization of materials were prepared; the bench was wiped with 70% alcohol to maintain an aseptic environment. The underside of each slide was labelled with the isolate code using permanent marker. Then, a small circle was made at the centre underside of each slide. For each sample, a drop of bacterial suspension was placed and spread on the glass slide. The smear was allowed to air-dry on the flat surface. After drying, the slide was then passed twice or thrice through the Bunsen burner flame to kill the bacteria and attach the sample to the slide. The slides were allowed to cool before staining.

2.12. Gram Stain Procedures

Gram staining was performed as described in the following procedures:

The slides were flooded with crystal violet (primary stain). After one minute, the slides were rinsed with tap water. Next, the slides were flooded with iodine (a mordant) that binds to the crystal violet. The mordant property of iodine prevents it from exiting the Gram-positive peptidoglycan cell wall. After one minute, the slides were rinsed gently with tap water. Then, the slides were flooded with an acetone-isopropanol mixture used as decolouriser to remove stains from the Gram-negative cells. After about 10-15 minutes, the slides were rinsed gently with tap water. Next, the slides were flooded with Safranin (counter-stain). After 10 minutes, the slides were rinsed with tap water. Then, the slides were drained and allowed to air-dry in an upright position. Finally, the slides were examined under oil immersion (1000x times magnification) using a bright field compound microscope.

2.13. Catalase Test

2.13.1. Quality Control

S. aureus subsp. *aureus* ATCC™ BAA-976™ obtained from IIUM-MC was used as positive control. A clinical isolate of group B *Streptococcus* also obtained from IIUM-MC was used as negative control.

2.14. Procedures

The catalase test was performed under the procedures listed below:

Grease pencil, sterile pipette, slides, hydrogen peroxide, inoculating loop, permanent marker, Bunsen burner, matches, and 70% alcohol for sterilization of materials were prepared. The working bench was wiped with 70% alcohol to maintain an aseptic environment. A clean glass slide was divided using grease pencil into two sections: one section was labelled 'test' and the other 'control'. A drop of bacterial suspension was placed on each section using a sterile pipette, and the bacterial suspension was gently smeared. Immediately, a drop of 3% hydrogen peroxide solution was added to the smear of the section labelled 'test' using a sterile pipette. The control smear ('control') was left without the addition of 3% hydrogen peroxide solution. The slides were observed with naked eyes for immediate formation of bubbles indicating the presence of catalase and production of oxygen.

2.15. Coagulase Test

2.15.1. Quality Control

Staphylococcus aureus subsp. *aureus* ATCC® BAA-976™ obtained from IIUM-MC was used as positive control. *Staphylococcus saprophyticus subsp. Staphylococcus epidermidis* ATCC 12228 also obtained from IIUM-MC was used as negative control.

2.16. Procedures

Each coagulase test was performed following the procedures listed below:

Sterile tubes, inoculating loop, permanent marker, Bunsen burner, matches, and 70% alcohol for sterilization of materials were prepared. The working bench was wiped with 70% alcohol to maintain aseptic environment. Three test tubes were prepared per test and labelled as 'test', 'positive control', and 'negative control'. One in five dilution of human plasma in normal saline (0.85% NaCl) was prepared. Each test tube was filled with 0.5 mL of the diluted human plasma. Whilst human plasma is preferred for detection of clumping factor with staphylococcus, it is not recommended for routine testing, as it may contain antibodies against staphylococcus. To the test tubes labelled 'test', two to three drops of bacterial suspension was added. To the test tube labelled 'positive control', two to three drops of known bacterial suspension (*Staphylococcus aureus* subsp. *aureus* ATCC® BAA-976™) was added. To the test tube labelled 'negative control', two to three drops of known bacterial suspension (*Staphylococcus epidermidis* ATCC 12228) was added. All the tubes were incubated at 37°C for 24 h. Each tube was observed hourly for clot formation by slowly tilting the tube at a 90° angle from the vertical. Some staphylococci produce fibrolysin, which could lyse the clot [14]. Presence of the clot after tilting indicates positive results. In the absence of clot after 24 h of incubation, incubation time was extended to 34 h at the same temperature. Any degree of clotting that remains after tilting the tube is recorded as positive result. On the contrary, absence of any degree of clotting is recorded as coagulase-negative result. After 24 h of incubation, the tubes were observed for fibrin clot formation.

2.17. Antibiogram of the Collected Isolates

To determine the antibiograms of the collected isolates, antimicrobial susceptibility testing was performed for all 40 confirmed MR-CoNS isolates identified using the methods described above. To accomplish this, all confirmed MR-CoNS isolates were subjected to the Kirby-Bauer disc diffusion method on Mueller Hinton agar (Oxoid, Basingstoke, England), as described in document M100-S25 of the Clinical and Laboratory Standards Institute (CLSI) [15]. The nine antibiotics used and their disc contents are shown in Table 2.

2.18. Preparation of Mueller-Hinton Agar

2.18.1. Procedures

Mueller Hinton agar, 500 mL measuring cylinder, spatula, electronic balance, 500 mL Duran bottle, distilled water, marker, and 70% alcohol for sterilization were prepared. The bench was wiped using 70% alcohol to maintain aseptic environment. Thirty-eight grams of Mueller Hinton agar media was suspended in 1 L of distilled water, and mixed thoroughly until completely dissolved. The mixture was sterilized in an autoclave at 121°C for 15 min. The freshly prepared medium was poured into sterile disposable petri dishes to a depth of 4mm±0.05mm. This corresponds to 20 mL medium volume for plates with a diameter of 100 mm. The agar medium was allowed to cool at room temperature and stored in the refrigerator at 8°C.

Table 2. Nine antimicrobial drugs and their disc contents in µg/mL.

S/N	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

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

2.19. Disc Diffusion Test

2.19.1. Procedures

Inoculating loop, fresh Mueller Hinton agar media, overnight MR-CoNS culture media, antimicrobial discs, sterile swabs, permanent marker, Bunsen burner, matches, and 70% alcohol for sterilization of materials were prepared. The working table was wiped with 70% alcohol to ensure aseptic environment. Mueller-Hinton agar plates and antibiotic discs were taken from the refrigerator and allowed to equilibrate to room temperature before use (approximately one hour).

A sterile swab was immersed in the prepared bacterial suspension; the swab was rotated against the side of the test tube to remove the excess fluid. The entire surface of the Mueller Hinton agar plate was streaked using the swab with samples or controls in at least three directions. To ensure that the entire surface of the agar was covered by the normal saline, the swab was streaked at least three times; rotating the plate at a 90° angle after each streak. Finally, the swab was passed around the edge of the plate. The plates were allowed to dry for 5-10 min at room temperature, covered.

Within 15 min after inoculation, antimicrobial discs were placed individually onto the surface of the inoculated agar plate with sterile forceps. Each disc was pressed down gently to ensure complete contact with the agar surface. Because the study involved 9 antimicrobial agents, two plates were used for each isolate: one plate with 5 antimicrobial discs, and another plate with 4 antimicrobial discs. Then, the plates were incubated at 35±2°C for 24 h.

After incubation, the diameters of the zones of complete inhibition were measured using a ruler to the nearest millimetre. Principally, zone margins should be read as the area showing no obvious growth as detected by unaided eyes. Finally, the results were interpreted following zone size criteria recommended by CLSI. The isolates were classified as Multidrug Resistance (MDR) if they were resistant to ≥ 3 non- β-lactam antibiotics as recommended by Al Laham [13].

2.20. E-Test for Determination of MIC of Oxacillin

2.20.1. Preparation of Standardized Inoculum

Standardized inoculum was prepared as recommended by the Minimum Inhibitory Concentration (M.I.C.) Evaluator (Oxoid) manual as described below:

Normal saline was taken from the fridge and left to attain room temperature. Next, a sterile 3 mL Pasteur pipette was used to transfer 3 mL of normal saline into prepared test tubes. Then, the test tubes containing normal saline and a pair of forceps were sterilized in the autoclave for 15 min at 121˚C. After that, the tubes were left to cool at room temperature. The quality control organisms used were: *Staphylococcus saprophyticus* obtained from IIUM-MC as positive control. And a reference strain of *Staphylococcus aureus* subsp. *aureus* American Type Culture Collection ATCC™ BAA-976™ also obtained from IIUM-MC was used as a negative control.

Three to 5 well-isolated colonies of both the test organisms (CoNS) and control organisms (*Staphylococcus aureus subsp. aureus* ATCC™ Business Associate Agreement BAA-976™ and *S. saprophyticus*) were selected from overnight nutrient agar plate cultures and suspended in 3 mL of normal saline (0.9%). Turbidity of the bacterial suspension was adjusted to be equivalent to a 0.5 McFarland standard.

2.21. Procedures for Performing the E-Test

The inoculating loop, fresh Mueller Hinton agar media, overnight MR-CoNS culture media, E-test strips, sterile cotton swabs, permanent marker, Bunsen burner, match, and 70% alcohol for sterilization of materials were prepared. The working table was wiped using 70% alcohol to maintain an aseptic environment. Mueller-Hinton agar plates were prepared and stored in the refrigerator until the time of use. On the day of the test, the Mueller Hinton agar plates and E-test strips were taken from the refrigerator and allowed to equilibrate to room temperature before use. This was done to ensure that condensation did not collect on the strip which may result in a disturbance of the gradient.

Within 15 min of preparation of standardized inoculum, a sterile cotton swab was immersed in the prepared bacterial suspension; the sterile cotton swab was then dipped into the inoculum and pressed against the edge of the tube to remove excess moisture. The entire surface of the Mueller Hinton agar plate of both the samples and the quality controls were inoculated by swabbing. To ensure that the entire surface of the agar had been covered by the normal saline, the swab was streaked at least three times; rotating the plate at a 90-degree angle after each streak. Finally, the swab was passed around the edge of the plate. Before applying the M.I.C.E, the plates were allowed to dry completely for 5-10 min at room temperature, covered. This was done to prevent excess moisture from distorting the gradient.

To avoid pre-growth of the organism, the oxacillin E-test strips were centrally placed on the agar surface with sterile forceps within 15 min after inoculation. To accomplish this, sterile forceps was used to remove the M.I.C.E from the sachet by handling the end with the logo and antibiotic code. The end with the lowest concentration was placed onto the plate first with the scale facing upwards, and antibiotic gradient downwards in contact with the agar; then, the strip was rolled carefully onto the agar to ensure good contact with the entire length of the M.I.C.E. After that, the strip was pressed down gently to ensure complete contact with the agar surface. Finally, all the plates were incubated in an inverted position under conditions appropriate for *Staphylococcus* growth: at 37°C in aerobic conditions for 24 hours.

2.22. Micro-Broth Dilution Test for Determination of Vancomycin MIC

Vancomycin MIC was determined by broth micro-dilution method, as described by Sharma, et al. [3] and Murray, et al. [14].

2.23. Quality Controls

Staphylococcus epidermidis ATCC 12228 and a *Staphylococcus aureus* subsp. *aureus* ATCC™ BAA-976™ (Thermo Scientific) reference strain were both obtained from IIUM-MC and used as positive and negative controls, respectively.

2.24. Procedures

2.24.1. Preparation of Stock and Working Solution

Both the working solution and stock solution were prepared as per instructions given in the product handling instructions sheet (TargetMol):

One mL of sterile distilled water was added into the original vial containing 100 mg of vancomycin hydrochloride powder and mixed completely by vortexing. This resulted in the initial concentration as shown below. One hundred mg of vancomycin hydrochloride powder was dissolved in 1 mL sterile distilled water, resulting in an initial concentration of 100 mg/mL. To prepare a 128 µg/mL working solution following the method of Sharma, et al. [3] the initial and final concentrations were input into the equation below for determination of the volume of stock concentration to use:

$$M_1V_1 = M_2V_2.$$

Where,

M_1 = Initial concentration of the stock solution (100,000 µg/ mL).

V_1 = Volume of stock needed (µL).

M_2 = Required final concentration of the working solution (adopted from Sharma, et al. [3] 128 µg/mL).

v_2 = Required final volume of working solution (4µL).

To obtain V_1, the above data was substituted into the above formula, and V_1 was calculated to be 0.00512 mL (5.12 µL). To calculate the amount of distilled water to be added to make up the final volume of the working solution, V_1 was subtracted from M_1, and 3995 µL was obtained. Therefore, to prepare the working solution, 5.12 µL of 100 mg/mL stock solution of vancomycin hydrochloride and 3995 µL sterile distilled water were added into a 15 mL falcon tube and mixed completely by vortexing.

2.25. Preparation of Inoculum and Inoculation

Two rows of tubes were prepared: the first tube of the first row was labelled 128 µg/mL, followed by 8 decreasing concentrations of 64, 32, 16, 8, 4, 2, 1, and 0.5 µg/mL. This resulted in a series of doubling dilutions with concentrations ranging from 64 to 0.5 µg/mL. The first tube contained 128 µg/mL of vancomycin hydrochloride working solution. A second row of 8 tubes labelled 1-8 for each sample was also prepared. To the first row, 1 mL of distilled water was added in each of the tubes: from tube of 16 up to tube 0.5.

Then, serial dilution was carried out with respect to the above dilution factor.

To the second row, 900 µL of fresh nutrient broth was introduced into each tube. Then, 1 mL of the serially diluted antibiotic from the first row was introduced into the respective tubes of the second row. Then, 50 mL of overnight nutrient broth culture with concentration of 10^5 Colony Forming Unit CFU/mL (prepared by diluting to a 0.5 McFarland suspension) was introduced into each tube.

Finally, the cultures were shaken and incubated at 37°C for 24 h. The values of the broth micro-dilution were interpreted following recommendations in document M1000-S25 of the CLSI.

3. RESULTS

3.1. Sample Collection and Distribution of MR-CONS from Different Clinical Sample

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 3. 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 4 and Table 5.

Table 3. Gender distribution of the collected isolates.

Gender	Number	Percentage
Male	26	65%
Female	14	35%

Table 4. 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 5. Distribution of collected samples from different wards of IIUM-MC.

Wards	Number	Percentage (%)
Intensive care unit (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 6.

The distribution of collected isolates according to diagnosis (of various infectious diseases) is shown in Table 7.

3.2. Re-identification of the MR-CoNS Isolates

Before further processing, all 40 isolates collected were re-identified using conventional biochemical methods to confirm them as MR-CoNS. The methods used in the re-identification process were morphology observation through subculture and gram staining, catalase test, and tube coagulase test. 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. See Figure 1.

Table 6. 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

Table 7. Distribution of collected MR-CoNS isolates from inpatients diagnosed with different infectious diseases.

S/No	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	Urinary tract infections (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

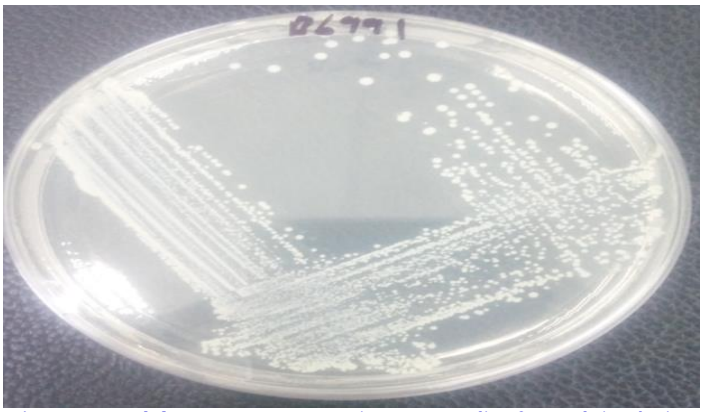

Figure 1. *Staphylococcus* grown on nutrient agar media after 24 h incubation under aerobic condition. Colonies are medium-sized, smooth, raised and glistening, with a grey to deep golden yellow colour.

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. Figures 2, 3 and 4 show the results of gram stain, catalase test and tube coagulase test, respectively.

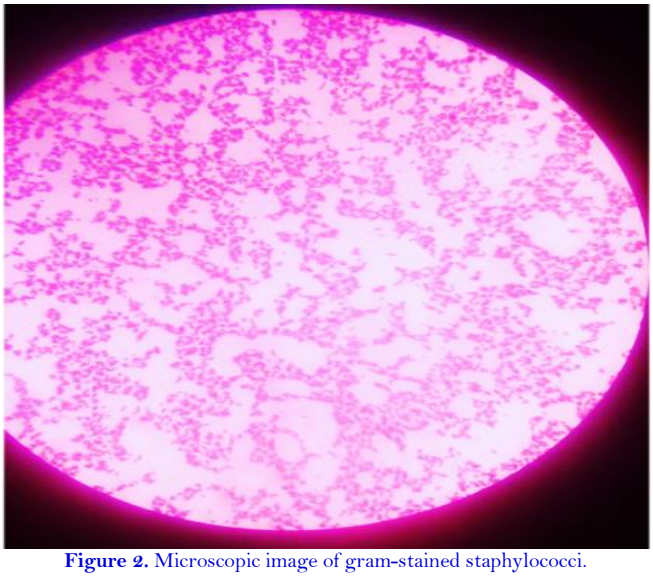

Figure 2. Microscopic image of gram-stained staphylococci.

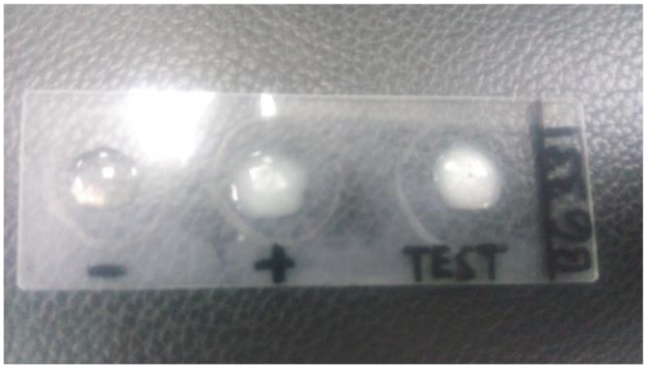

Figure 3. Catalase test.

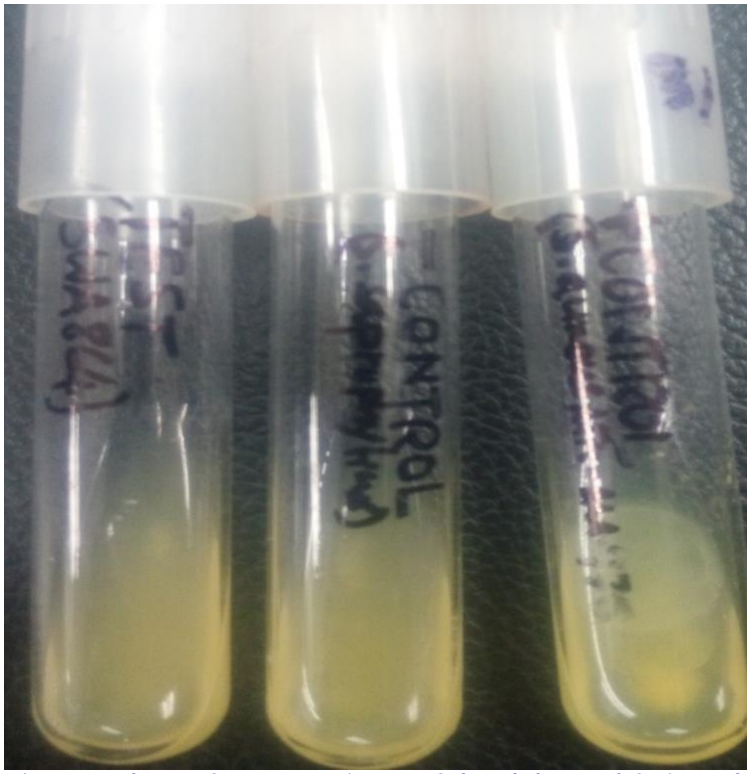

Figure 4. Tube coagulase test: negative control showed absence of clotting, and positive control showed presence of clotting.

Therefore, all 40 isolates were proven to be gram-positive, catalase-positive, and coagulase-negative cocci.

3.3. Antimicrobial Susceptibility Test

Twenty-nine MR-CoNS isolates from IIUM-MC and 11 MR-CoNS isolates from HTAA were examined for antimicrobial susceptibility profiles against 9 antibiotics commonly used to treat MR-CoNS infections using the disc diffusion method. This panel of antibiotics and their respective concentrations (µg/mL) were: oxacillin (30 µg/mL), linezoid (30 µg/mL), teicoplanin (30 µg/mL), clindamycin (2 µg/mL), erythromycin (15 µg/mL), ciprofloxacin (5 µg/mL), trimethoprim-sulfamethoxazole (1.25/23.75 µg), ceftaroline (30 µg/mL), and vancomycin (32 µg/mL). 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 document M1000-S25 of National Committee for Clinical Laboratory Standards (NCCLS). The zones of inhibition of one of the 40 samples are shown in Figure 5 and Figure 6.

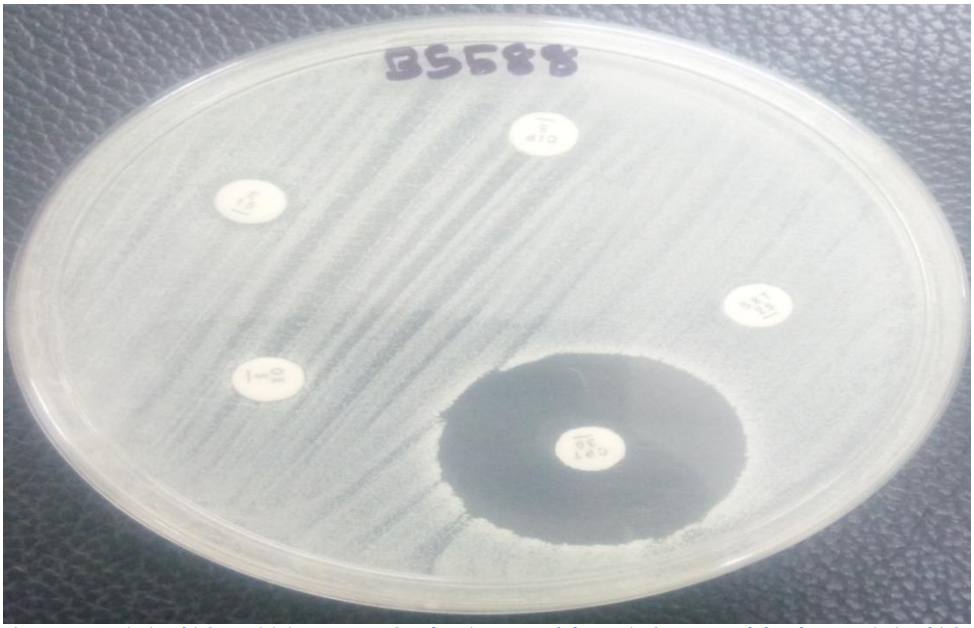

Figure 5. Antimicrobial sensitivity test results showing one of the 40 isolates tested for the 5 antimicrobial drugs using Kirby and Bauer method.

Figure 6. Antimicrobial sensitivity test results showing the same isolate tested for another 4 antimicrobial drugs using the Kirby and Bauer method.

The antibiograms of all the categories of isolates such as blood, tissues, and swabs are shown in Table 8 and Figure 7.

Table 8. 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	-	-	-	-	-	-
Vancomycin[b]	32 µg/mL	0	0%	0	0%	40	100%

Note: [a, b] 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.

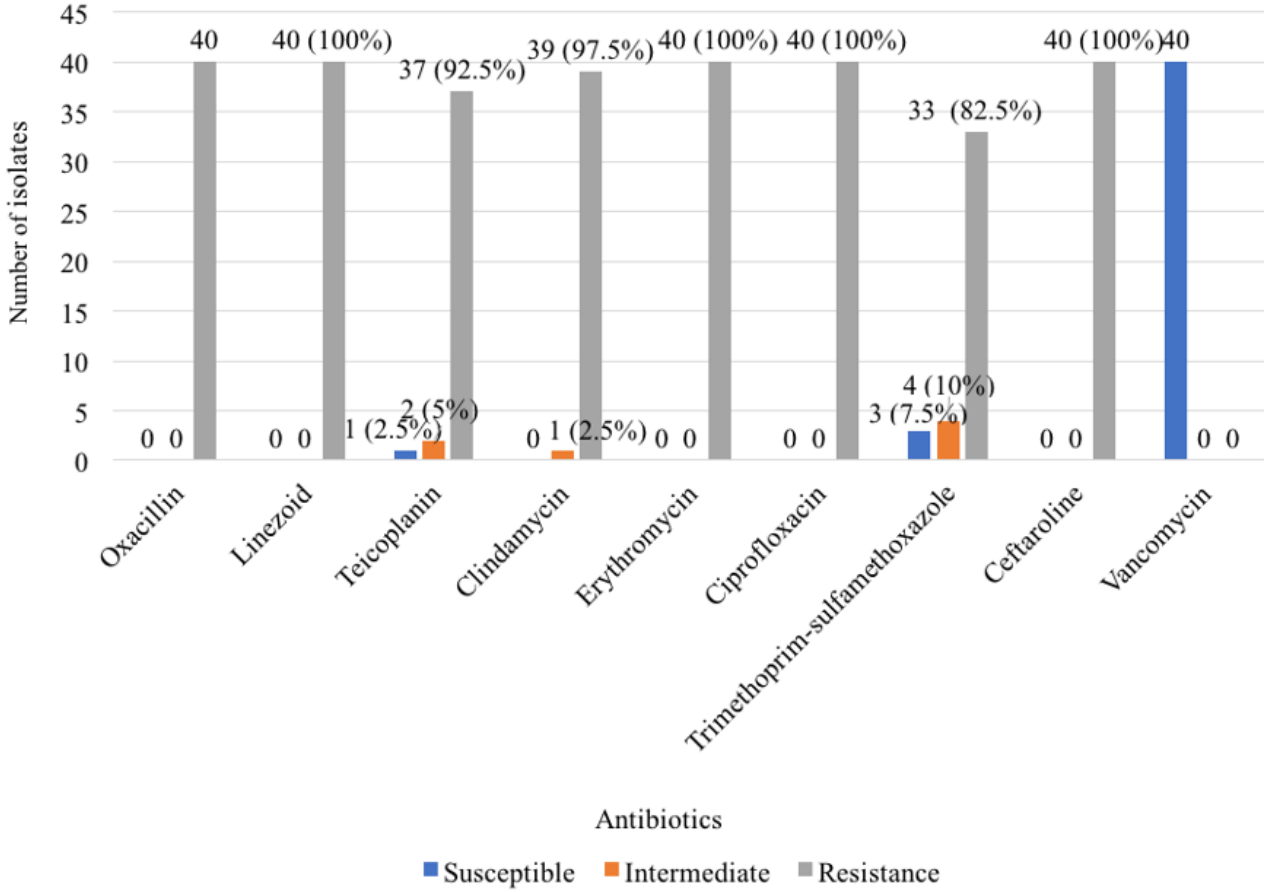

Figure 7. Antibiograms of MR-CoNS in all categories of isolates: blood, tissue, and swabs. The highest antimicrobial resistance was observed against linezoid, erythromycin, ciprofloxacin, and ceftaroline, while the least resistance was observed against trimethoprim-sulfamethoxazole.

The isolates were classified as MDR if they were resistant to more than 3 non-β-lactam antibiotics [13]. The five predominant MDR antibiotic resistance profiles are shown in Table 9. MDR profiles of the specific categories of isolates of MR-CoNS are shown in Table 10.

Table 9. 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

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

Table 10. 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%

3.4. Oxacillin MIC in MR-CoNS

To confirm methicillin resistance, oxacillin MIC (30 µg/mL) was determined for all 40 MR-CoNS isolates by using the E-Test**,** and preliminary results 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.

3.5. Interpretation of the Results

As described in the principle of the E-Test, the results 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 as described below:

If the growth touches the strip on the white section, the MIC was read as the value in that section. See Figure 8.

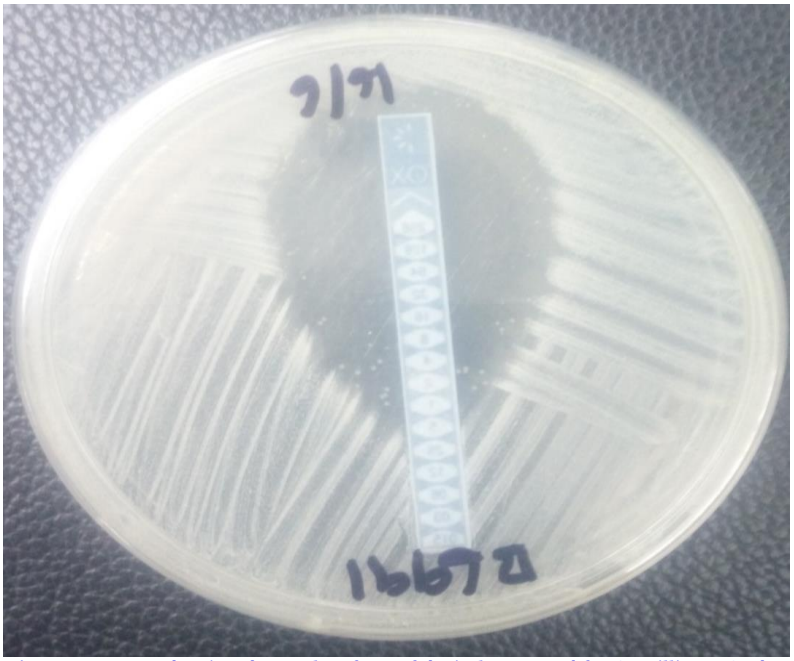

Figure 8. E-Test showing the results of one of the isolates tested for Oxacillin MIC where the growth touched the strip in the white section.

If the growth touches the strip in the black section, MIC was read as the value in the next white section above where the growth has finished. See also Figure 9.

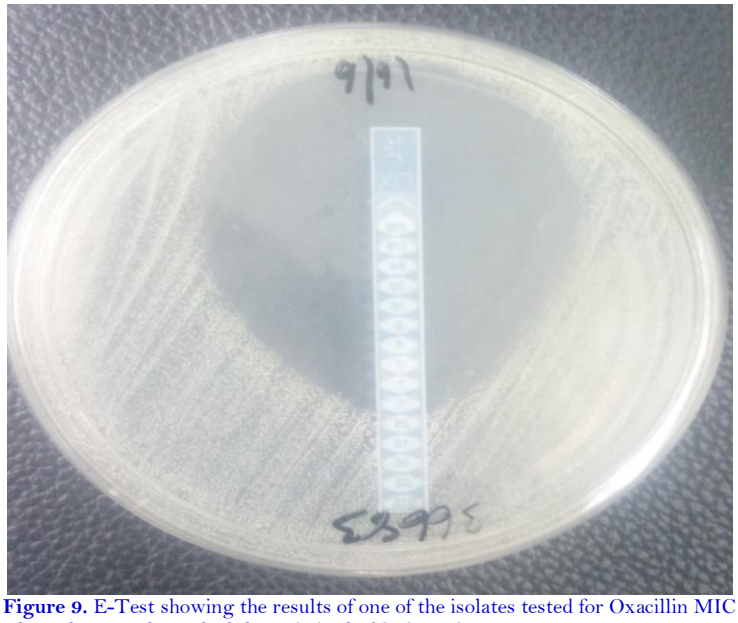

Figure 9. E-Test showing the results of one of the isolates tested for Oxacillin MIC where the growth touched the strip in the black section.

If there was a growth along the entire length of the strip (No zone of inhibition), the MIC was read as greater than the highest value on the strip. See also Figure 10.

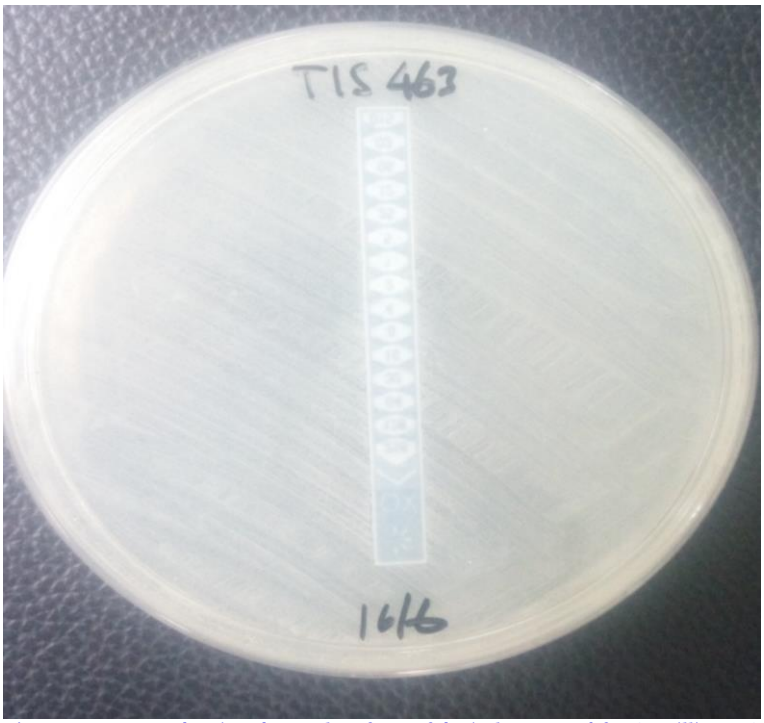

Figure 10. E-Test showing the results of one of the isolates tested for Oxacillin MIC but didn't show any zone of inhibition.

For greater zones of inhibition where growth of the organism does not intersect with the strip, the MIC should be read as less than the lowest value of the strip. However, this large zone of inhibition was not observed in our study.

Overall, the results were expressed as susceptible and resistant; as per the recommendations given in document M1000-S25 of CLSI. These preliminary results were further interpreted according to the recommendations given in document M1000-S25 of the CLSI as shown in Table 11.

Table 11. MIC of Oxacillin in MR-CoNS.

Antimicrobial drug	Disc content (µg/mL)	Interpretative categories and MIC breakpoints (µg/mL)		
		Susceptible (S)	Intermediate (I)	Resistant (R)
Oxacillin	30 µg/mL	≤ 0.25	-	≥ 0.5

Thirty-eight MR-CoNS isolates were oxacillin-resistant, as their MIC values were ≥0.5 µg/mL, and the remaining 2 isolates were oxacillin-susceptible, as their MIC values were ≤ 0.25.

3.6. MIC of Vancomycin in MR-CONS

Likewise, to confirm the susceptibilities of MR-CoNS to vancomycin by the disc diffusion method, broth micro-dilution test was performed as recommended by Sharma, et al. [3] and Murray, et al. [14]. *S. epidermidis* ATCC 12228 (vancomycin susceptible) and *S. aureus* ATCC 43300 (vancomycin susceptible) were used as positive controls, as described by Murray, et al. [14] and Jain, et al. [16]. After overnight incubation at 37°C for 24 h as recommended by Murray, et al. [14] the results of broth micro-dilution were interpreted according to the recommendations given in document M1000-S25 of CLSI as shown in Table 12.

Table 12. MIC of vancomycin in MR-CoNS.

Antimicrobial drug	Disc content (µg/mL)	Interpretative categories and MIC breakpoints (µg/mL)		
		Susceptible (S)	Intermediate (I)	Resistant (R)
Vancomycin	30 µg/mL	≤ 4	8-16	≥ 32

According to the above recommendations, the final results showed all 40 MR-CoNS isolates as vancomycin susceptible, with MIC ranging from 1-4 µg/mL as shown in Figure 11.

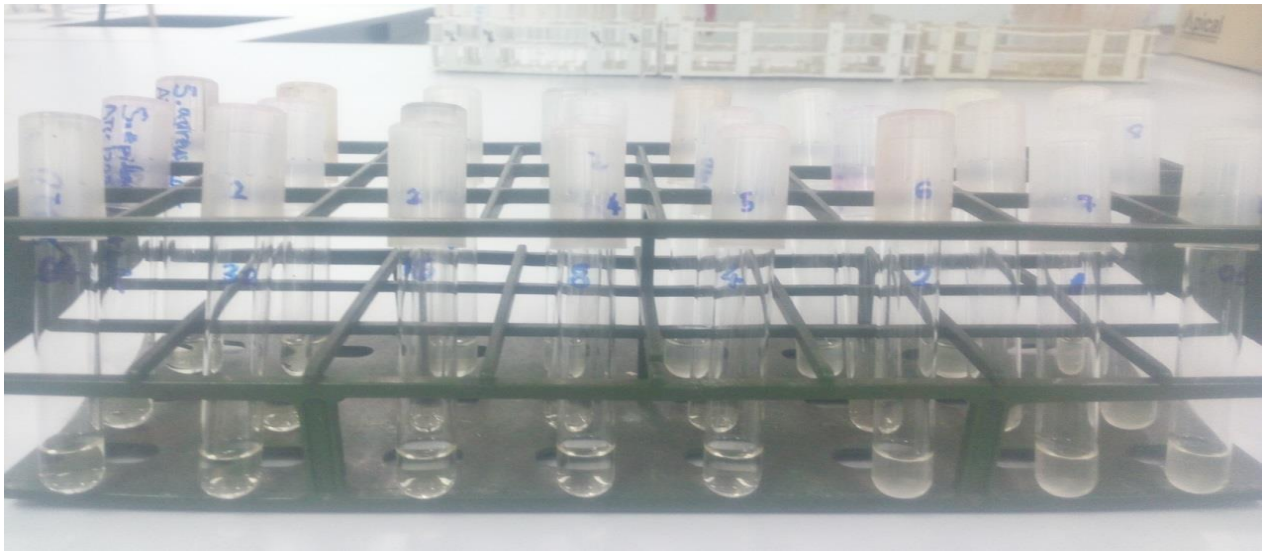

Figure 11. The first row shows the MIC of vancomycin for one of the 40 isolates. The second and third rows show the MIC of vancomycin for vancomycin-susceptible controls (*S. epidermidis ATCC 12228* and *S. aureus* ATCC 43300). For the isolate (first row), from right to the left, 0.5-2 µg/mL of vancomycin did not inhibit bacterial growth, while 4 µg/mL was the lowest concentration that inhibited bacterial growth, followed by 8 µg/mL, 16 µg/mL, and 32 µg/Ml.

4. DISCUSSION

Although there are about 33 species of CoNS, only few of them have been associated with an increase in nosocomial infections, especially due to the use of indwelling medical devices such as orthopaedic prostheses, valvular prostheses, central and peripheral venous catheters, pace-makers, and artificial heart valves [17-19].

The distribution of CoNS antibacterial resistance against a panel of commonly used antibacterial drugs in this study is presented in Table 8. 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. [10]. Resistance to erythromycin and ciprofloxacin in this study was higher than Sharma, et al. [3] (27.9% and 36.3% respectively). Contrasting results were reported by Al Tayyar, et al. [20] and Gilani, et al. [21] who found higher susceptibility levels of 100% and very low resistance levels of 2% to linezolid, respectively. In Turkey, Alicem, et al. [22] also reported 100% susceptibility to linezolid.

In our 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. [10] who reported more than 80% resistance to teicoplanin. In contrast, Deyno, et al. [23] reported low resistance levels of 50% and 11% to trimethoprim-sulfamethoxazole and clindamycin, respectively. Our results were in contrast to the findings of Al Tayyar, et al. [20] 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 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. [3] (48%), and almost equal to values reported by Becker, et al. [6]; Alicem, et al. [22] (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. [3] and Becker, et al. [6]. However, there have been some emerging cases of vancomycin intermediate-resistance reported in Germany [17] Turkey [22] Italy [24] Australia [25] and US Garrett, et al. [26]. Jain, et al. [16] reported a 15% decrease in vancomycin susceptibility in 127 CoNS isolates, and Bugum, et al. [19] reported vancomycin resistance in some *S. haemolyticus* strains. The findings reported by Bugum, et al. [19] 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 [25]. 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 9 and Table 10. 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 [8] in Germany [17] and in India [18]. Meanwhile, Kitti, et al. [27] reported high levels of multi-drug resistance against 7-10 antibiotics in MR-CoNS. Recently, a study conducted in the US revealed a moderately high level of multi-drug resistance of 73.5% in MR-CoNS [28]. In general, these studies attribute the increase of multi-drug resistance to surgical prophylaxis, exposure to multiple antibiotics, and indiscriminate use of antibiotics.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. 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 the treatment strategies in particular. This study investigated antibiogram 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, the results show that MR-CoNS were 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.

5.2. 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 the genes such as *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.

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 this study and reported in previous studies, healthcare providers should adhere to both preventive and control measures against the horizontal spread of resistance.

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Competing Interests: The author declares that there are no conflicts of interests regarding the publication of this paper.

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