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Antibiotic resistance properties of *Staphylococcus epidermidis* isolated from hospitals in Selangor

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ABSTRACT

A total of 89 Coagulase-Negative Staphylococci (CoNS) samples used in this study were collected from clinical hospitals in Selangor. These isolates were grown on Mannitol Salt Agar (MSA) to screen for pink colonies that do not reduce mannitol which is a characteristic of CoNS. The purified isolates were subjected to standard biochemical tests which include Gram stain, slide coagulase, catalase, and urease test. Identification of *Staphylococcus epidermidis* was performed using the *tuf* gene sequencing method which confirmed the species at a total of 60 out of the 89 isolates. When tested against several antibiotics, 41.7% of the isolates were found to be resistant to cefoxitin followed by erythromycin (38.3 %), gentamicin (16.7 %), rifampin (16.7 %), clindamycin (15.0 %), and ciprofloxacin (8.3 %). In contrast, all of the *S. epidermidis* isolates were sensitive against linezolid. This supports the use of linezolid in the current treatment of *S. epidermidis* infections. Hence, the speciation of *S. epidermidis* and its antibiotic resistance patterns may further establish their role as a significant pathogen and help in initiating proper antimicrobial therapy.

Keywords: *S. epidermidis, tuf gene sequencing, antibiotic, linezolid*

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INTRODUCTION

Hospital or nosocomial infection is a serious concern worldwide. From every hundred hospitalized patients, this infection can latch onto every seven or ten patients in developed and developing countries respectively [1]. Bacteria holds the most significant causative agents [2] causing about 90% of these infections [3]. One of the main causes of hospital infections is a group of staphylococcal called Coagulase Negative Staphylococci (CoNS) [4,5].

Generally, staphylococci are recognized as a group of Gram-positive cocci, non-motile and non-spore-forming bacteria, that may appear single, in pairs, tetrads, and even in 'grape-like' clusters [6]. Unlike Coagulase Positive Staphylococci (CoPS) which include *Staphylococcus aureus*, CoNS is a group of staphylococci that can be characterized by the absence of coagulase enzyme. Among the important members of this group includes *S. epidermidis, Staphylococcus saprophyticus,* and *Staphylococcus haemolyticus* [7]. Like *S. aureus*, CoNS regularly populate the skin and mucous membranes of humans and animals [8]. However, while *S. aureus* is known to be pathogenic, any infections involving CoNS are generally brushed off as insignificant contaminants and they are often taken for granted. However, there has been increasing evidences that this group of *Staphylococcus* is able to cause various types of infections reported worldwide [4,5,9,10]. In Malaysia, a study in a teaching hospital reported that 33.0 % of CoNS was isolated from blood cultures as compared to only 10.4 % *S. aureus* isolated [11]. Other reports on infections caused by CoNS include bloodstream [4,10], wound [12], urinary tract, skin and soft tissue, prosthetic implant, and various other indwelling device-related infections [13]. As such, the significance of this group of *Staphylococcus* in a medical setting is increasing and requires further attention.

S. epidermidis is the most dominant species in CoNS [2,14,15]. As a common colonizer or commensal of the skin [16,17], *S. epidermidis* has evolved into a significant opportunistic pathogen [18]. This bacterium was reported to cause infections like prosthetic valve endocarditis [15], wound infection [19], and also known to be among the major cause of infections such as bloodstream infection [20] and neonatal septicaemia [21]. The higher risk group for *S. epidermidis* infections are neonates, immunocompromised individuals, hospitalized patients [22], and individuals with indwelling medical devices [23], which are mostly centralized in healthcare settings. Simultaneously with the diverse infections, there is an issue on the antimicrobial resistance of this species of *Staphylococcus* [4,12,24].

The history of antibiotic resistance in *S. epidermidis* goes way back to the 1940s. During the early 1940s, the first natural antibiotic, penicillin, was introduced for use in healthcare to treat general infections [25,26]. Shortly after, penicillin-resistant strains of *S. aureus* were isolated in 1942 from hospitalized patients in the US [27]. In 1949, a penicillin-resistant strain of *S. epidermidis* was isolated in the US from three fatal cases of subacute bacterial endocarditis [28]. Following that, semi-synthetic penicillin called methicillin was introduced in 1959 to replace

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penicillin [29,30]. However, the bacteria subsequently developed resistance against methicillin as well. Methicillin-resistance *S. aureus* or MRSA was first reported in the UK from nephrectomy wounds and finger infection cultures in 1961 [31]. In the same year, the first methicillin-resistant strain of *S. epidermidis* was also isolated from children hospitalized in a pediatric hospital in the UK [32].

Nowadays, the antibiotics used in the treatment of *S. epidermidis* infections include rifampin (ansamycin), linezolid (Oxazolidinones), vancomycin (glycopeptides), and quinupristin/dalfopristin (streptogramins) [33,34]. Clindamycin (lincosamides) is also used for the treatment of staphylococcal skin and soft tissue infections [13]. However, there have been several reports on the resistance of this species of staphylococci against multiple types of antibiotic classes like penicillins, aminoglycosides, fluoroquinolones, and macrolides [2,12,15]. This frequency of antibiotic resistance in *S. epidermidis* demonstrates the misuse and overuse of antibiotics [35]. As a result, infections of this bacterium render difficult to treat due to the risk of antibiotic-resistant nature [36,37].

In Malaysia, studies on *S. epidermidis* in hospital settings is lacking as most of the time this *Staphylococcus* species remain unidentified as CoNS. The negligence may contribute to the extent of the actual impact of *S. epidermidis* infections in hospitals. Hence, this study was conducted to identify *S. epidermidis* from CoNS isolated from various clinical samples and to investigate the antibiotic resistance properties of this bacterium. It is hoped that the data obtained from this study may provide information for the framework of management therapy against infections caused by *S. epidermidis*.

EXPERIMENTAL

Bacterial isolation and maintenance

A total of 89 presumptive CoNS samples from various clinical settings such as blood, pus, and wound swabs, were collected from the pathology department of some clinical hospitals in Selangor. The presumptive CoNS samples were first grown on MSA (Oxoid, UK), a standard media used to isolate CoNS from mannitol fermenting *S. aureus*. Following that, the CoNS isolates were streaked repeatedly on Brain Heart Infusion (BHI) agar (Oxoid, UK) to obtain pure cultures. These pure CoNS cultures were maintained in 20 % glycerol stock at -80 ˚C and subcultured on fresh BHI broth when needed.

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S. epidermidis identification

The pure cultures of CoNS were further subjected to standard biochemical tests which include Gram stain, catalase, slide coagulase, and urease to pre-determine *S. epidermidis* among the isolates [38–41]. The identity of the presumptive *S. epidermidis* isolates was later confirmed via amplification of the *tuf* gene sequencing method. The genomic DNA was first extracted using DNeasy Blood & Tissue Kits (Qiagen) according to the manufacturer's instructions.

Amplification of the *tuf* gene was performed using tuf-F (5'- GCC AGT TGA GGA CGT ATT CT- 3') and tuf-R (5'- CCA TTT CAG TAC CTT CTG GTA A-3') which amplifies 412 bp of the 1185 bp *tuf* gene [42]. The PCR reaction mix was prepared using MyTaq Red Mix (Bioline) in a total volume of 50 µL: 25 µL of 2X MyTaq Red Mix buffer, 2 µL of 10 µM of each primer, and 5 µL of DNA as a template. The PCR condition was as follows: 1 cycle of 95 ˚C for 15 minutes; followed by 35 cycles of 95 ˚C for 30 seconds, 56 ˚C for 30 seconds, and 72 ˚C for 45 seconds; and a final step of 72 ˚C for 10 minutes [42,43]. The amplicons were analyzed in 1.8 % agarose gel electrophoresis at 90 V for 80 minutes using *S. epidermidis* ATCC 12228 as a positive control. The PCR products were sent for sequencing to Bio Basic Asia Pacific Pte Ltd (Singapore) using the forward primer. The resulting sequence data was used to interrogate the nucleotide collection of the Genbank database (https://blast.ncbi.nlm.nih.gov/Blast) using the Basic Local Alignment Search Tool (BLAST) algorithm.

Antimicrobial susceptibility test

The antimicrobial susceptibility of the *S. epidermidis* clinical isolates was tested using the Kirby-Bauer disc diffusion method on Mueller Hinton Agar (MHA) [44]. The 60 isolates were first grown on Mueller Hinton Broth (MHB), overnight at 37 ˚C at 180 rpm [45]. On the following day, the broth was diluted at 1: 100 in fresh MHB and further incubated for three to four hours, to achieve the log phase. The turbidity of the cultures was then adjusted to 0.5 McFarland standard which is equivalent to 1 X 10^8 cfu/mL, at OD_{625} nm between 0.08 to 0.13 [46]. The adjusted culture was streaked on MHA plates and the antibiotic discs were placed on each of the plates before incubating at 37 ˚C for 18-24 hours [47].

The isolates were tested against cefoxitin (FOX, 30 µg), ciprofloxacin (CIP, 5 µg), clindamycin (CLI, 2 µg), erythromycin (ERY, 15 µg), gentamicin (GEN, 10 µg), linezolid (LZD, 30 µg), and rifampin (RIF, 5 µg) (Oxoid, UK) [48]. These antibiotics were chosen based on their targets and classes as shown in Table 1 as recommended by the Clinical and Laboratory Standards Institute (CLSI), while *S. aureus* ATCC 25923 was used as a control strain. The activity of each S. epidermidis isolates against the seven antibiotics was measured by the diameter of zone of inhibition and interpreted as according to CLSI 2018 guidelines [49].

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Table 1: Selected antibiotics, their classes and references

Antibiotic	Classes	Target	Reference
Cefoxitin	Penicillins	Inhibits cell wall synthesis	[50,51]
Ciprofloxacin	Fluoroquinolones	DNA synthesis inhibitors	[4,51]
Clindamycin	Lincosamides	Protein synthesis inhibitors (Inhibit 50s subunit)	[4,51]
Erythromycin	Macrolides	Protein synthesis inhibitors (Inhibit 50s subunit)	[4,13]
Gentamicin	Aminoglycosides	Protein synthesis inhibitors (Inhibit 30s subunit)	[4,51]
Linezolid	Oxazolidinones	Protein synthesis inhibitors (Inhibit 50s subunit)	[4,13]
Rifampicin	Ansamycins	RNA synthesis inhibitors	[4,51]

RESULTS AND DISCUSSION

Identification of S. epidermidis isolates

The characteristics of the positive control *S. epidermidis* ATCC 12228, negative control *S. aureus* ATCC 25923, and a representative CoNS isolate on MSA are visible in Figure 1.

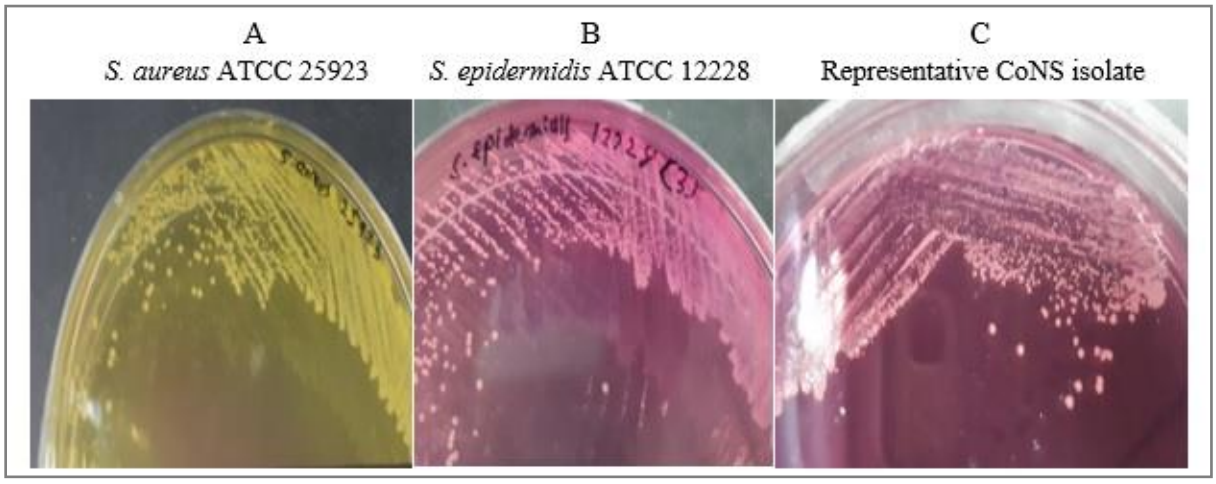

Figure 1: Characteristics of *S. aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228) and a representative CoNS isolate on MSA. *S. aureus* is a mannitol fermenter where the acidic by-products will reduce phenol red to yellow colour. In contrast, *S. epidermidis* does not ferment mannitol, thus the agar remains red in colour [52].

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The purified CoNS isolates were then subjected to standard biochemical tests [53]. Gram stain was performed to rule out Gram-negative bacteria, catalase test to rule out *Streptococcus*, while the slide coagulase test was conducted to rule out Coagulase Positive Staphylococci (CoPS). The urease test was performed to rule out urease negative isolates, as *S. epidermidis* is known to be urease positive [53]. Some of the results are shown in Figure 2.

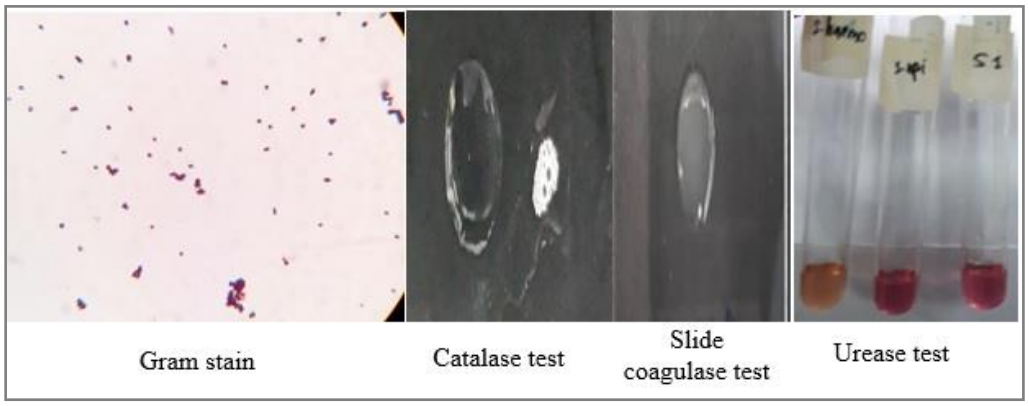

Figure 2: Results of the biochemical tests on representative CoNS isolates. The isolates stained purple on Gram stain, show bubbles formation on catalase test, no clumping on slide coagulase test, and displayed bright pink of fuchsia colour in urea broth.

PCR of the *tuf* gene was performed to confirm the identity of the presumptive *S. epidermidis* isolates. Figure 3 displays the results for *tuf* gene sequencing for some of the isolates with 412bp amplicons.

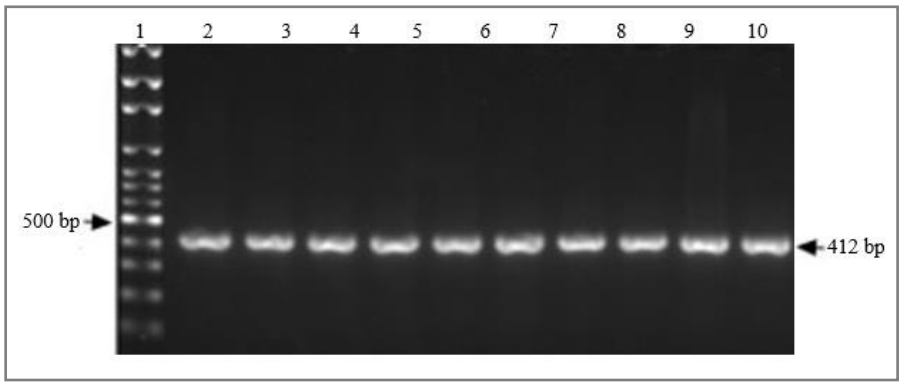

Figure 3: Amplification of the *tuf* gene. Lane 1: 100 bp ladder; Lane 2: positive control *S. epidermidis* ATCC 12228; Lane 3-10: Isolates B12; B13; B14; B16; B17; B19; B20 and B21 respectively.

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The purified PCR products were then sequenced and the results of a representative *S. epidermidis* isolates can be seen in Figure 4 with 100 % of identity. From the 89 samples of CoNS, a total of 60 of the isolates were identified as *S. epidermidis*.

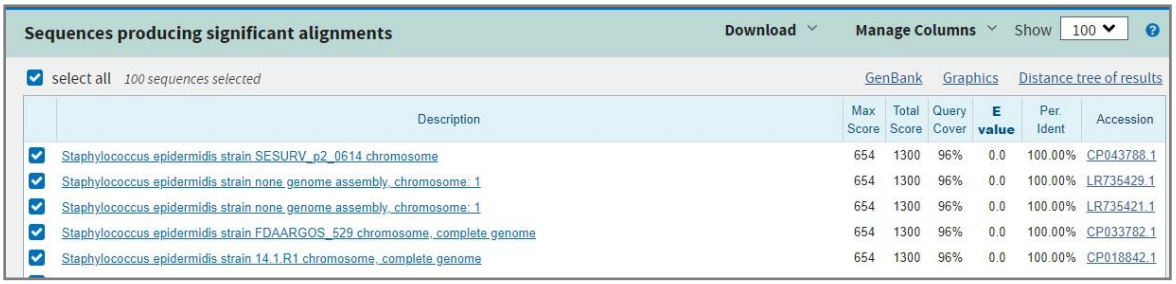

Figure 4: Interrogation of a representative *S. epidermidis* isolates on the Genbank database using BLAST algorithm

Antibiotic resistance patterns of the S. epidermidis isolates

These isolates were further subjected to antibiotic susceptibility testing by using the Kirby-Bauer disc diffusion method as shown in Figure 5.

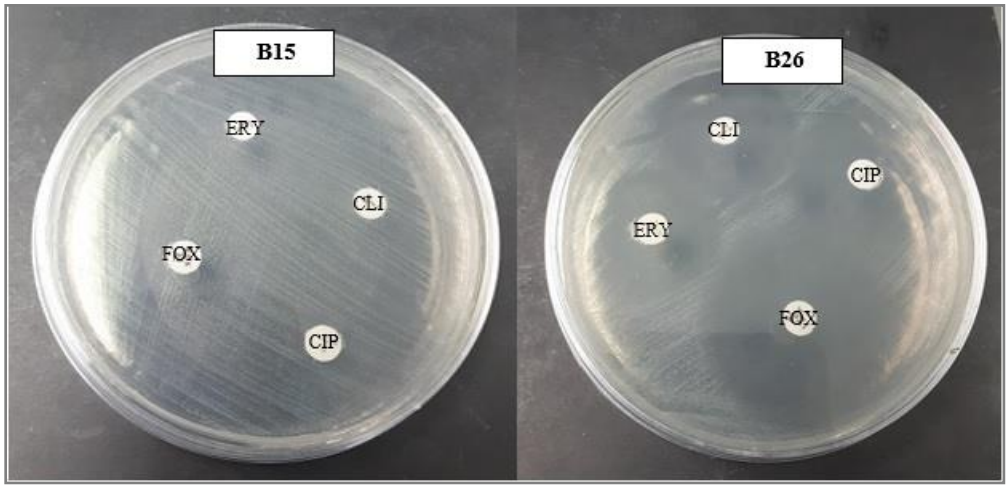

Figure 5: Results of a representative *S. epidermidis* B15 and B26 against selected antibiotics based on the Kirby Bauer disc diffusion method. The results display the activity of both B15 and B26 against four antibiotics which were cefoxitin (FOX), ciprofloxacin (CIP), erythromycin (ERY) and clindamycin (CLI). B15 was resistant to all the four antibiotics while B26 was found to be susceptible to all the four antibiotics.

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The summary of the resistance patterns of the *S. epidermidis* isolates against the seven antibiotics is shown in Figure 6. In general, the clinical isolates of *S. epidermidis* were found to display a various range of resistance against all antibiotics except for linezolid. The highest percentage of resistance was observed in cefoxitin, whereby at 41.7 %, almost half of the isolates were resistant to this antibiotic. This is followed by erythromycin, a macrolide whereby resistance was observed in 38.3 % of the isolates. At 16.7 %, similar resistance was recorded against gentamicin and rifampicin while 15.0 % and 8.3 % of the *S. epidermidis* isolates were found to be resistant against clindamycin and ciprofloxacin respectively. In contrast, all the isolates were found to be susceptible to linezolid.

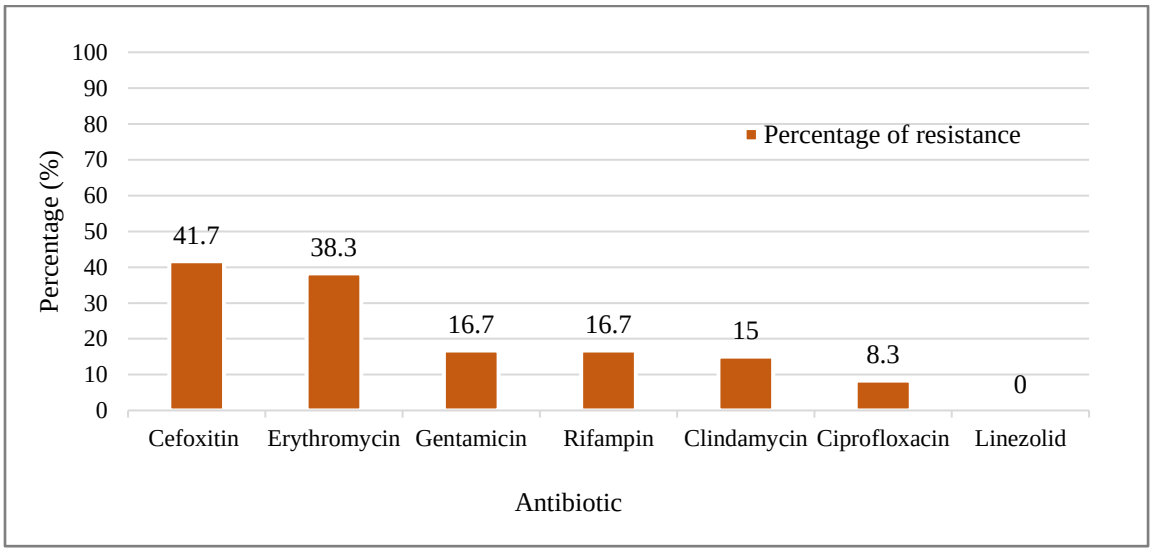

Figure 6: Resistance patterns of *S. epidermidis* against different antibiotics. The antibiotics and their classes are cefoxitin (penicillins), erythromycin (macrolides), gentamicin (aminoglycosides), rifampin (ansamycins), clindamycin (lincosamides), ciprofloxacin (fluoroquinolones) and linezolid (Oxazolidinones).

The high resistance against cefoxitin in clinical *S. epidermidis* isolates was in agreement with studies conducted from hospitals in India and Iran [2,36]. Similarly, resistance against erythromycin by *S. epidermidis* was also reported from a hospital in Iran [2]. These findings question the eligibility of these antibiotics to be used in the treatment of *S. epidermidis* infections. The pattern of resistance of *S. epidermidis* against cefoxitin, erythromycin, and gentamicin was generally similar to a study conducted in a teaching hospital in Malaysia in 2014 [54]. This resistance frequency is also in agreement with reports that this bacterium is commonly resistant to a group of antibiotics like penicillins, macrolides, and aminoglycosides [2,12,15].

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None of the clinical *S. epidermidis* isolates were found to be resistant against linezolid. This is similar to studies conducted on clinical *S. epidermidis* isolates in India and Italy [13,55]. This finding is also in agreement with the claim of the significance of linezolid in the treatment for *S. epidermidis* infections [33], whereby it is used for treatment in cases of glycopeptide-resistant infections [29,56]. However, there were also isolated cases on resistant strains of *S. epidermidis* when tested against linezolid in India and Saudi Arabia [15,36]. So, to maintain the efficiency of linezolid as one of the therapeutic agents against *S. epidermidis*, it should be a reserve drug that must be used prudently [13,35].

CONCLUSION

In this study, a total of 60 clinical *S. epidermidis* were successfully isolated and identified. The antibiotic susceptibility testing showed the highest percentage of resistance against cefoxitin (41.7 %) followed by erythromycin (38.3 %), gentamicin (16.7 %), rifampicin (16.7 %), clindamycin (15.0 %), and ciprofloxacin (8.3 %). Meanwhile, all the isolates were sensitive against linezolid, which demonstrates the need for linezolid to be a reserve drug for *S. epidermidis* infections that should be used prudently. Therefore, the speciation of *S. epidermidi*s and its antibiotic resistance patterns may further establish their role as a significant pathogen and help in initiating proper antimicrobial therapy based on the resistance pattern.

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