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Unmasking Bioflim- producing Gram Positive Cocci in Post-Operative Wound Infections

Dr. Mrunal M. Patwardhan[1*], Dr. Pankaj Joshi[2], Dr. Neeta Jangale[3]

[1]Assistant professor Department of Microbiology Govt medical college Miraj, Maharashtra, India
[2]Associate professor Department of Microbiology Govt medical college Miraj, Maharashtra, India
[3] Professor and Head of Department of microbiology, GMC Miraj Maharashtra, India

*Corresponding Author: Dr. Mrunal M. Patwardhan Email: dr.mrunal28@gmail.com

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Abstract: **Introduction**-The third most frequent nosocomial infection to be documented is surgical site infection. They are responsible for the rising expense, morbidity, and death rates associated with surgery. The risk of surgical site infections has ranged from 2.5% to 41.9%. Staphylococcus aureus is common from wound infections during surgery, which might act as stimulants for the development of systemic infections. **Objectives**-The objective of this study was to isolate and identify various Gram-positive cocci from surgical site infected cases and detect their antimicrobial susceptibility pattern along with their capability of biofilm production. **Materials and Methods-** A total of 166 culture positive gram-positive cocci were isolated from infected surgical sites. Standard procedure and protocol were followed for identification of isolates. Antimicrobial susceptibility testing was done by Kirby Bauer disc diffusion method as per current CLSI guidelines. Methicillin resistance in staphylococcus isolates was tested by cefoxitin disc diffusion method. Biofilm formation detection done by Congo red and tube method. **Results-** Out of 166 Gram positive cocci, 112 were *S. aureus* and 54 were *Enterococci. S. aureus* isolates showed highest percentage resistance to penicillin (95.53) and erythromycin (59.82%). Methicillin resistance was found to be 43.75 %. Enterococci isolates (including Enterococci spp. *and Enterococcus faecalis*) showed highest percentage resistance to penicillin (98.14%) followed by ampicillin (88.88%). Among the 112 isolates of *S. aureus*, a significant majority, 84 isolates (75%), were found to be biofilm producers, in contrast, of the 54 isolates of Enterococcus spp., 30 isolates (55.5%) were biofilm producers. **Conclusion**: This indicates that a higher proportion of *S. aureus* isolates are capable of producing biofilms compared to Enterococcus spp.

Keywords: Surgical site infection, Methicillin resistant *Staphylococcus aureus*, *Enterococcus*, Biofilm formation.

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INTRODUCTION

Surgical site infections are known to be one of the most common causes of nosocomial infections worldwide which accounts for nearly 20-25 % [1]. As per the Centers for Disease Control and Prevention, postoperative wound infection is defined as infections that are occurring within 30 days of surgery or within one year (i.e. secondary infection) of an operation at the site of incision [1].

These infections may be superficial or deep incisional infections. Globally, surgical site infection (SSI) rate varies from 2.5% to 41.9%. In India, the incidence of surgical site infection varies from 3.6% to 22.5% [2].

Surgical site infections contribute to a substantial rate of mortality, significant morbidity, considerable prolongation in the length of hospitalization, and added treatment expenses. The situation is more severe in developing countries like India, where resources are scarce and staff is always in short supply [2].

The most common organisms encountered in surgical site infections are-
Staphylococcus aureus, *Coagulase Negative Staphylococcus* (CONS), *Pseudomonas*, *E. coli*, *Klebsiella*, *Enterobacter*, *Proteus*, *Acinetobacter,* and *Enterococcus, etc [3].*

Inappropriate choice of antibiotics increases favouring emergence of pathogenic drug-resistant bacteria [4]. *Staphylococcus aureus* was susceptible to penicillin but due to misuse of antibiotics, it is resistant to most of the penicillin group worldwide due to methicillin resistance. Most Methicillin-resistant *Staphylococcus aureus* (MRSA) infections occur in health care settings and are called hospital-acquired MRSA [3].

According to WHO, out of all the cases reported for *Staphylococcus aureus*, 20% are of postoperative wound infection [1]. The most common microorganism responsible for causing postoperative wound infection is *Staphylococcus aureus*. It is an important cause of community and hospital-acquired surgical site infections [5].

It is now widely accepted that naturally, bacteria prefer to live in surface-associated communities called biofilms. In the biofilms, the bacteria are embedded in an extracellular polymeric matrix and are protected against environmental stresses, antimicrobial treatment, and the host immune system [6-8].

Therefor The aim of this study was biofilm production and antimicrobial susceptibility pattern in Gram positive cocci isolated from clinical samples of post-operative wound infection.

MATERIAL AND METHODS

Two-year descriptive study (June 2022 to May 2024) was conducted in tertiary care hospital in the department of microbiology government medical college Miraj. Total 166 samples were from various clinical specimens collected under universal aseptic precaution from the depth of wound by using two sterile swabs or pus and submitted to the laboratory for aerobic bacterial culture identification, antimicrobial susceptibility and their biofilm production. All Gram-positive cocci isolated from the clinical specimens from post operative wound (swab or pus) received in the Department of Microbiology government medical college Miraj were included. Any sample other than postoperative wound swab and pus and any bacteria other than Gram positive cocci isolated from postoperative wound swab and pus were excluded.

SPECIMEN PROCESSING

The samples included were post-operative pus and wound swabs. All the first swabs were used to make a smear on a clean, grease free slide and subjected to Gram staining by Modified Hucker's method. The second swab was immediately inoculated onto blood agar and MacConkey agar. The culture plates were incubated at 37°C overnight. All the biochemical tests were carried out using standard media. Antimicrobial susceptibility testing of all isolates was done by Kirby bauer disc diffusion method as per CLSI.[9] Biofilm detection for the biofilm producing bacteria done by Congo red agar and tube method.[10]

MRSA detection [9]- All isolated strains of *S.aureus* were tested for methicillin resistance by Kirby Bauer's disc diffusion method using cefoxitin (30µg) disc. An inhibition zone diameter of ≤ 21 mm was reported as methicillin resistant and ≥ 22 mm was considered as methicillin sensitive.

Detection of inducible clindamycin resistance (D-TEST) [9]: Mueller Hinton Agar plate was inoculated with staphylococcal bacterial suspension having turbidity equivalent to 0.5 McFarland's turbidity standard. Erythromycin (15 µg) disc was placed at a distance of 15mm (edge to edge) from clindamycin (2 µg) disk. Following overnight incubation at 37ºC, flattening of zone (D shaped) around clindamycin in the area between the two discs, indicated inducible clindamycin resistance.

Antimicrobial Susceptibility Test [9]: Antibiotic susceptibility was performed on Mueller Hinton agar following Clinical and Laboratory Standards Institute (CLSI) current guidelines. Antimicrobial agents like Penicillin (10U), Cefoxitin (30µg), Erythromycin (15µg), Clindamycin (2µg), Linezolid (30µg), Cotrimoxazole (25µg), Ciprofloxacin (5µg), Gentamicin (10µg) for *Staphylococcus aureus*.

Bile Esculin Test [11]: This test is used for the presumptive identification of Enterococcus species and group D streptococci. Organisms that are bile esculin–positive are able to grow in the presence of 40% bile and to hydrolyse esculin. Most Enterococcus species and group D streptococci will blacken bile esculin medium within 24 hours; rare strains may require a 48-hour incubation before hydrolysis is apparent.

6.5% NaCl Tolerance Test [11]: The salt tolerance test (6.5% NaCl broth) separates Enterococcus species from the group D non enterococcal streptococci (*S. gallolyticus* subspecies). Two or three colonies of organism to be identified was inoculated into a tube of nutrient broth with 6.5% NaCl and incubated at 35°C for 3 days. Growth was judged by the turbidity seen after dispersing any sediment. Enterococcus species will be salt-tolerant. *S. gallolyticus* subspecies will not grow in 6.5% NaCl broth.

Pyrrolidonylarylamidase Test (PYR) [11]: The PYR hydrolysis test is a presumptive test for both group A and group D enterococcal streptococci. It replaces the bacitracin test and the salt tolerance test for group A streptococci and Enterococcus species, respectively. The enzyme that is detected is called pyrrolidonyl arylamidase. Broth containing PYR (L-pyrrolidonyl-β-naphthylamide) was inoculated with the organism and incubated at 35°C for 4 hours. During this time, PYR is hydrolyzed. Free βnaphthylamide was then detected by addition of 1-2 drop of PYR reagent, N, N dimethylaminocinnamaldehyde. Development of red colour was considered as positive indicating hydrolysis of PYR.

Antimicrobial Susceptibility Test for enterococci [9]- All isolates were subjected to antimicrobial susceptibility testing on Mueller Hinton Agar by Kirby-Bauer disc diffusion method using commercially available discs. e.g. Penicillin(10u), Ampicillin (10), Erythromycin (15 µg), Vancomycin (30 µg), Linezolid (30 µg), Ciprofloxacin (15 µg), Gentamicin (120µg).

Biofilm detection methods [10]-

Tube Method for Biofilm Detection [10]

Bacterial isolates were inoculated into 10 mL of Tryptic Soy Broth (TSB) supplemented with 1% glucose and incubated aerobically at 37°C for 24 hours. After incubation, the broth was carefully decanted, and the tubes were gently washed three times with phosphate-buffered saline (PBS; pH 7.2) to remove planktonic bacteria. The tubes were then air-dried and stained with 0.1% crystal violet for 10–15 minutes. Excess stain was removed by rinsing the tubes with distilled water, and the tubes were allowed to dry in an inverted position. Biofilm formation was assessed by observing the presence of a visible violet film lining the walls and bottom of the tube. A thick, adherent film indicated strong biofilm production, a thin but visible film indicated moderate biofilm production, whereas the absence of a visible film or the presence of only a ring at the air–liquid interface was considered negative for biofilm production

Congo Red Agar (CRA) Method for Biofilm Detection [10]

Biofilm production was also assessed using the Congo Red Agar (CRA) method. Congo Red Agar was prepared by supplementing Brain Heart Infusion (BHI) agar with 5% sucrose and Congo red dye (0.8 g/L). The test isolates were streaked onto the CRA plates and incubated aerobically at 37°C for 24–48 hours. Following incubation, colony morphology was examined. Black colonies with a dry, crystalline appearance were considered positive for biofilm (slime) production, whereas red, pink, or Bordeaux-colored colonies were interpreted as negative. Colonies showing intermediate darkening without a crystalline appearance were considered intermediate or weak biofilm producers.

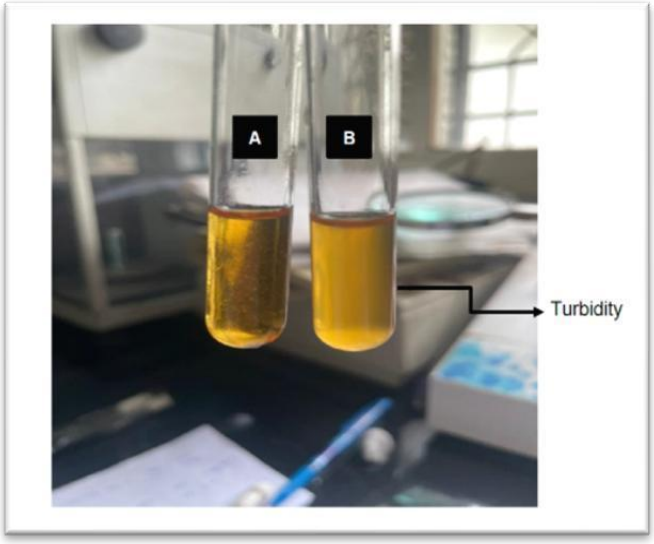

Fig 1- 6.5% NaCl for Enterococcus species turbidity seen in B tube

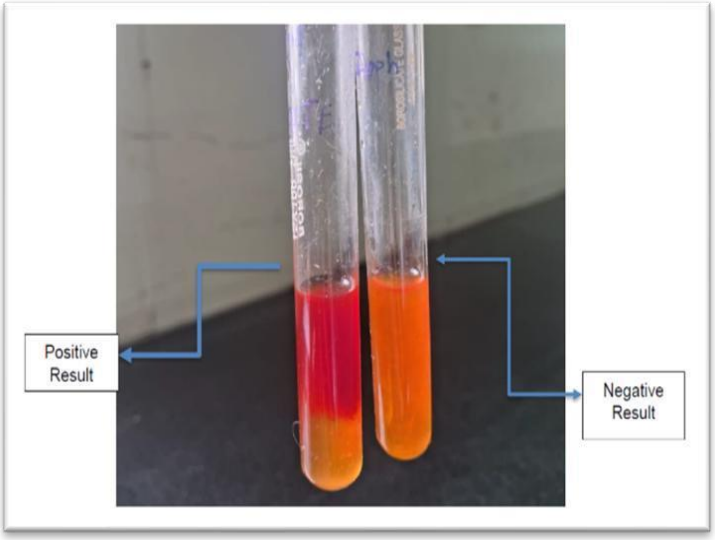

Fig 2 PYR test positive for enterococcus species and negative staphylococcus

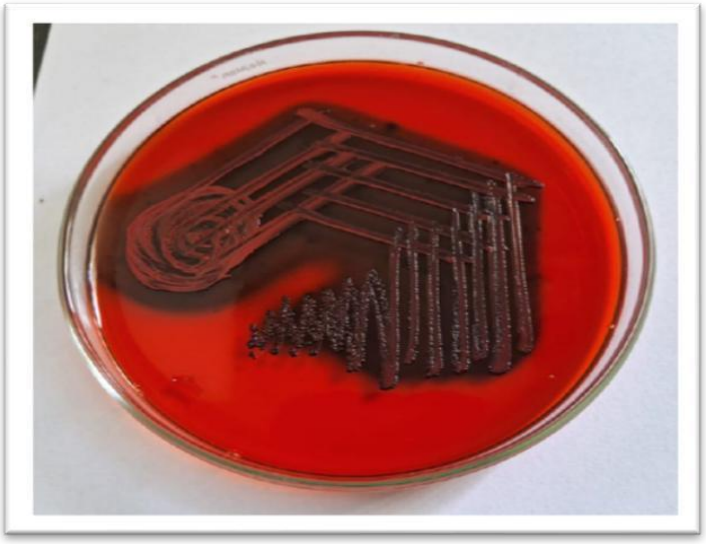

Fig 3- Biofilm production by Congo red agar method

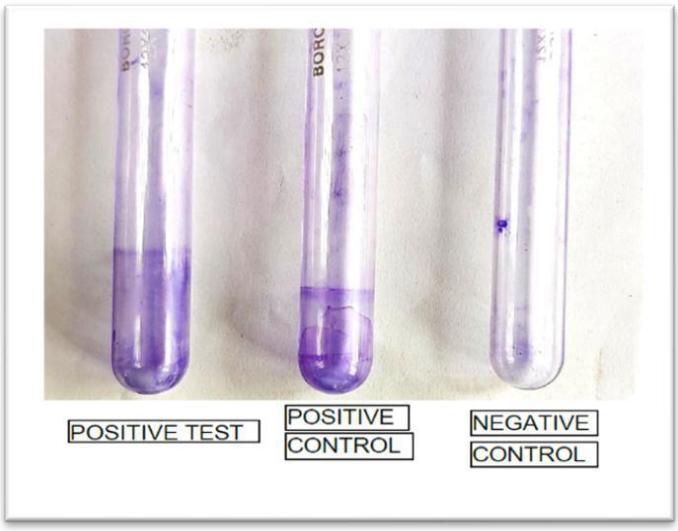

Fig 4 Tube method for detection of biofilm production

RESULTS

A total of 166 Gram positive cocci were isolated, out of which 72 (44%) were from post operative site pus and 94 (56%) were from post operative site swab. Out of 166 Gram positive cocci, 112 were *S. aureus* and 54 were enterococci. (chart-1)

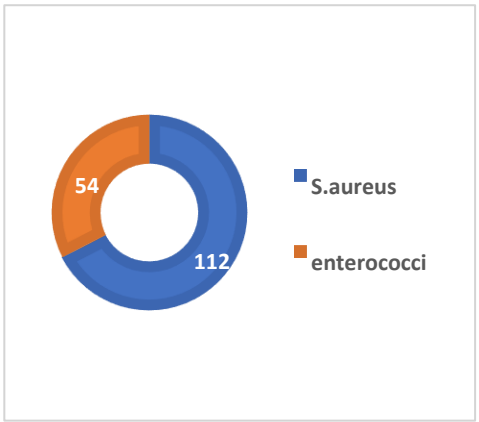

Chart-1

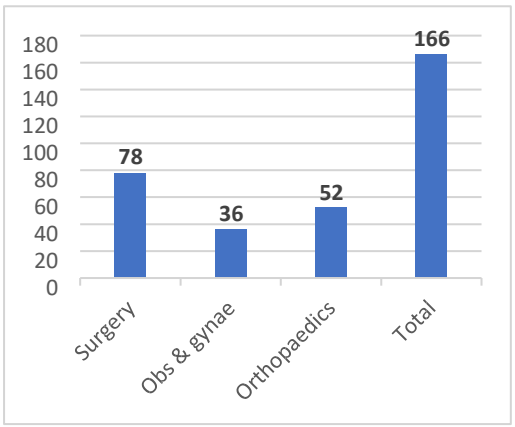

Chart-2

Gram Positive cocci isolates were more commonly obtained from specimens submitted by Surgery department (46.98%) followed by Orthopaedics department (31.32 %). (chart-2)

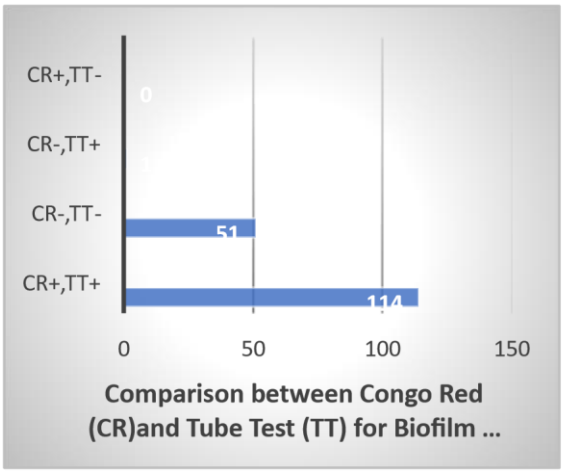

Chart-3

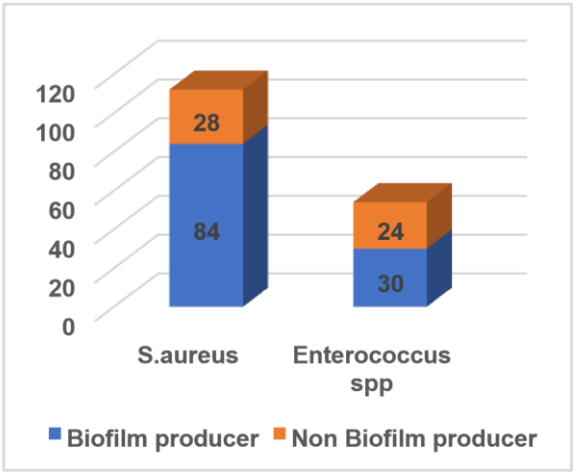

Chart-4

Total 114 (68.67%) Gram-positive cocci strains were biofilm producers and non-biofilm producing strains were 51 (30.72%). Out of 166 isolates, 114 isolates were positive by both methods. Only these isolates were considered as biofilm producers for further analysis. (Chart-3)

Among the 112 isolates of *S. aureus*, a significant majority, 84 isolates (75%), were found to be biofilm producers, while the remaining 28 isolates (25%) were non-biofilm producers. In contrast, of the 54 isolates of Enterococcus spp., 30 isolates (55.5%) were biofilm producers, and 24 isolates (44.4%) were non-biofilm producers. (Chart4)

S. aureus isolates showed highest percentage resistance to penicillin (95.53) and erythromycin (59.82%). Methicillin resistance was found to be 43.75 %. One isolate was resistant to linezolid. (Chart-5)

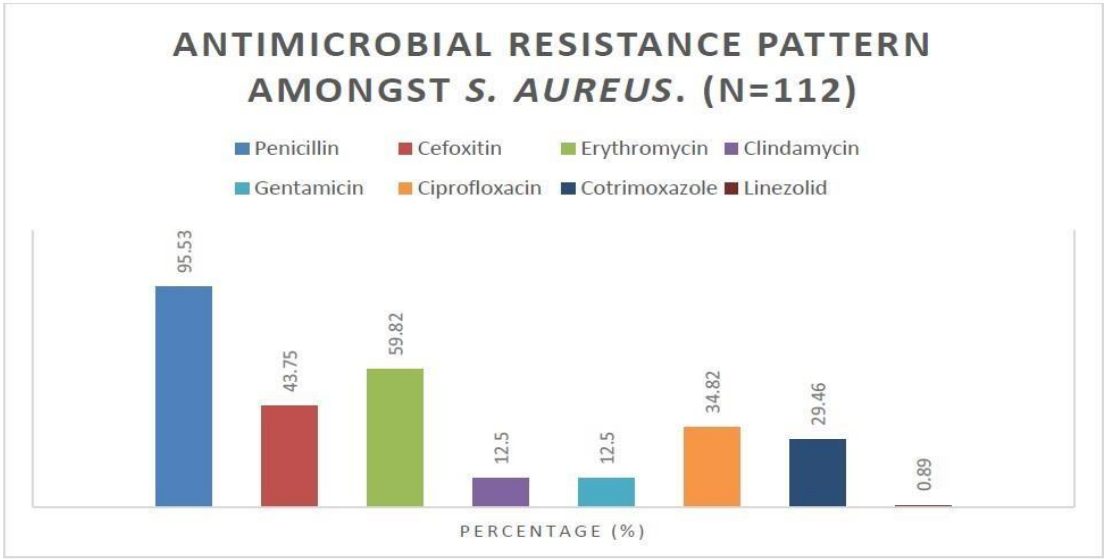

Chart-5

Enterococci isolates (including Enterococci spp. *and Enterococcus faecalis*) showed highest percentage resistance to penicillin (98.14%) followed by ampicillin (88.88%) and erythromycin (44.44%). One isolate was resistant to linezolid. (Chart-6)

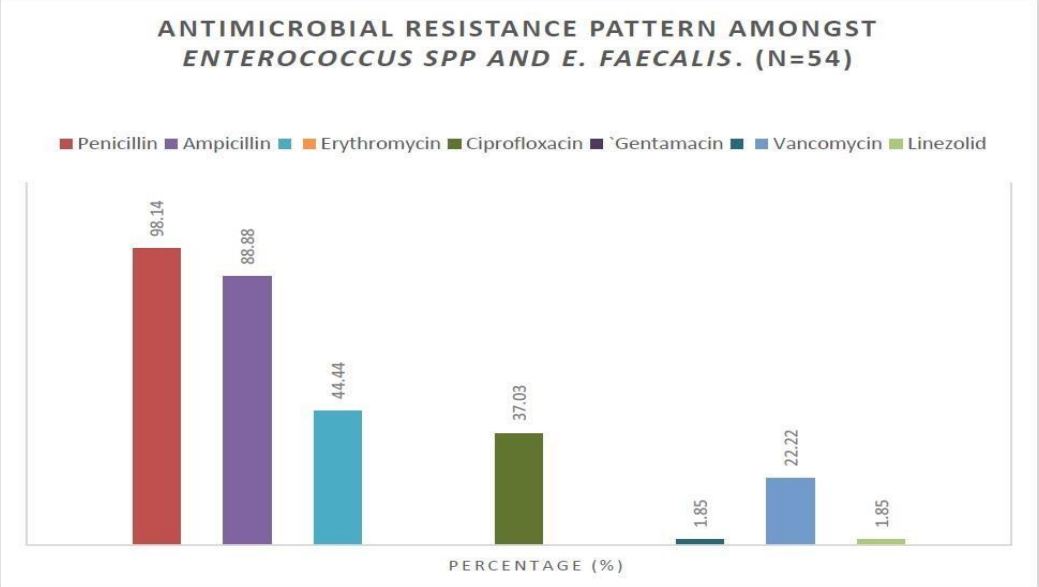

Chart-6

DISCUSSION

In our study we found *S.aureus* was predominant isolate which similar with study conducted by Mulu W, Kibru G *et al*. [12]. It is Suggested that *S. aureus* is most commonly associated with endogenous source because it resides on skin and nasal flora and also contamination from environment, surgical instruments or from hands of health care workers [13].

We also found that highest GPC from surgery (47%) followed by orthopedics (31%). A similar observation was made by Sanjana RK *et al*. [14].

We observed that out of total 166 Gram-positive cocci isolates, 114 (68.6%) were detected as biofilm producers by both Congo red agar and the tube method. Similar finding was observed by Pokharel K *et al*. [15].

Out of 112 isolates of *S. aureus*, a significant majority, 84 isolates (75%), were found to be biofilm producers. In contrast, out of the 54 isolates of Enterococcus spp., 30 isolates (55.5%) were biofilm producers. Similar findings were observed by Chen M *et al*. (2013) [16].

For *S.aureus* highest resistance was observed to Penicillin (95.53%) followed by Erythromycin 59.82%. In our study methicillin resistance was found to be 43.75%, which is comparable with the study by Mohanty *et al*. [17].

Among Enterococci the highest resistance was observed to Penicillin (98.14%), followed by Ampicillin (88.88%) and Erythromycin (44.44%). These findings are comparable with the findings of Suneja Surabhi et.al. (2023) [18].

Out of 112 isolates, 84 (75%) were biofilm producers, indicating that biofilm formation is a common virulence trait in the studied bacterial population. Among biofilm-producing isolates, 58 (69%) were identified as MRSA, suggesting a strong coexistence between methicillin resistance and biofilm-forming ability. Additionally, 95% of biofilm producers showed resistance to penicillin and 58% showed resistance to erythromycin, demonstrating markedly elevated multidrug resistance among biofilm-forming strains. These findings are comparable with the findings of Donlan RM *et.al*.[19].

This pattern suggests that biofilm formation significantly contributes to antimicrobial resistance, likely by limiting antibiotic penetration, reducing bacterial metabolic activity, and promoting persistence of resistant. The higher resistance rates to β-lactams (penicillin) and macrolides (erythromycin) among biofilm producers further support the role of biofilm as a protective niche that enhances survival under antibiotic pressure. The coexistence of MRSA with biofilm formation highlights a clinically important phenotype associated with persistent and difficult-to-treat infections.

Overall, these findings emphasize that biofilm-producing MRSA strains represent a high-risk group for therapeutic failure and chronic infection, underscoring the need for routine screening of biofilm formation and strict antimicrobial stewardship.

CONCLUSION

Antimicrobial resistance (AMR) is a growing concern in the management of surgical site infections (SSIs), often complicating treatment and leading to poorer patient outcomes. One significant factor contributing to this challenge is the formation of biofilms by various microorganisms. This biofilm formation not only enhances the bacteria's resistance to antibiotics but also makes it more difficult for the immune system to clear

the infection. To optimize clinical decision-making, it is crucial for microbiology laboratories to detect and analyze both the biofilm-forming capabilities and the antimicrobial susceptibility of Gram-positive cocci isolated from postoperative wound infections. This detailed information will enable clinicians to choose the most appropriate and effective therapies, ultimately improving patient outcomes and reducing the risk of persistent or recurrent infections.

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Conflict of Interest: The authors declare no conflict of interest.
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