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The Dual Threat: Phenotypic Characterization and Virulence Factor Detection of Staphylococci Across Various Clinical Samples

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KEYWORDS

Staphylococcus schleiferi, Biofilm, Microtitre plate assay, Infection control

ABSTRACT:

Background and aim: Coagulase-negative staphylococci (CoNS), apart from Staphylococcus aureus are major causative agents of nosocomial infections, as evident from their increasing incidence in recent years. Aim: To phenotypically characterize staphylococcal isolates from various clinical samples, detect different virulence factors and evaluate their antibiotic resistance pattern.

Materials and Methods: A total of 130 clinical specimens were analysed in a tertiary care hospital. The isolates were identified, speciated using standard methods, biofilm formation as well as antibiotic susceptibility using the Kirby Bauer disc diffusion method were determined in the department of Microbiology. Statistical analysis was done by counts and percentages using MS Excel version 2010.

Results: CoNS constituted the majority (67.5%) of isolates, while S. aureus accounted for 32.4%. A significant proportion of staphylococci (75.6%) were strong biofilm producers, with an additional 16.2% being moderate biofilm producers, highlighting the widespread nature of this virulence factor. High resistance was observed among biofilm-producing strains to conventional antibiotics with methicillin resistance of 91.6% in S. aureus and 84% in CoNS, although all isolates remained sensitive to vancomycin and linezolid. Staphylococcus schleiferi (20%) was the most common CoNS species identified, followed by S. hemolyticus (16%) and S. simulans (16%), a finding that differs from some other regional studies.

Conclusion: The multiple antibiotic resistance and pathogenic potential of biofilm producers emphasize the importance of developing simple, reliable, and inexpensive methods to identify virulence factors, determine the antibiotic sensitivity of CoNS and the critical need for rigorous infection control measures to mitigate the burden of these infections.

INTRODUCTION

Microbial biofilms represent complex communities of microorganisms encased in an extracellular polymeric matrix, attached to a surface. These structures are ubiquitously found in nature and play a critical role in human health, particularly in the context of infections. Coagulase negative Staphylococci are prominent components of the normal skin and mucosal microflora but have simultaneously emerged as leading causative agents of healthcare-associated (nosocomial) infections over the past two decades. The clinical significance of staphylococci is largely attributed to their increasing recognition for biofilm formation, a characteristic that significantly contributes to treatment failure and poses a serious challenge to healthcare systems. It is estimated that 65-80% of all human infections are biofilm-related, encompassing a wide array of conditions from medical device infections to chronic wound infections.[1] The ability of staphylococci, including both *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS), to form biofilms is a major virulence factor that allows these pathogens to persist within the host, evade immune responses, and withstand antibiotic therapy. CoNS, traditionally regarded as commensals or contaminants, have increasingly been recognized as uniquely qualified pathogens and are responsible for 9% of nosocomial infections. They cause infections in immunocompromised hosts, especially patients with

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cancer, end-stage renal disease, renal transplantation, burns, and infections associated with indwelling catheters, shunts, and prosthetic devices. This contributes to prolonged hospital stays, increased healthcare costs, and higher morbidity and mortality rates.[2] The phenotypic characterization of staphylococcal isolates, coupled with an evaluation of their biofilm production and antibiotic resistance profiles, is therefore essential for understanding the local epidemiology, informing empirical treatment guidelines, and developing effective infection control strategies.[3]

The present study was undertaken to isolate and phenotypically characterize staphylococci from various clinical samples, and to evaluate their ability to produce biofilms. This study also focuses on the clinical implications, antibiotic resistance trends, diagnostic challenges, and current prevention and treatment strategies, thereby contributing to the broader understanding and management of staphylococcal biofilm-related infections.

METHODS

Study Design and Sample Collection This study involved the collection of a total of 130 different clinical specimens (e.g., blood, urine, wound swabs, device tips) over a period of one year from both inpatients and outpatients received in the Department of Microbiology, were included in the study after taking informed consent from the patients. CoNS mixed with other isolates were excluded from this study.

Bacterial Isolation and Identification Upon receipt, the clinical specimens were inoculated onto Blood agar and MacConkey's agar plates. These plates were then incubated overnight at 37°C to facilitate bacterial growth. Initial identification of colonies suspected to be staphylococci was performed using standard microbiological techniques. These included colony morphology, Gram staining to determine cell morphology, followed by biochemical tests such as the catalase test, slide coagulase test, tube coagulase test and DNase test. [4-6]

Speciation of Coagulase-Negative Staphylococci (CoNS) For isolates identified as CoNS, further speciation was carried out using a series of biochemical tests as described by Kloos and Schleifer (1975). These tests included the Voges-Proskauer test, evaluation of hemolysin production, nitrate reduction, detection of urease, ornithine decarboxylase activity, and fermentation of various sugars. This comprehensive panel allowed for the identification of different CoNS species, which is crucial given their varying pathogenic potentials. [7]

Antibiotic Susceptibility Testing (AST) Antibiotic susceptibility profiling was performed using the Kirby-Bauer disc diffusion method, a widely accepted phenotypic method for assessing antimicrobial resistance. The antibiotics (Hi-media, Mumbai, India) selected for testing were representative of commonly used agents in clinical practice. These included Penicillin (10 IU), Gentamicin (10 µg), Ciprofloxacin (5 µg), Cotrimoxazole (1.25/23.75 µg), Cefoxitin (30 µg), Erythromycin (15 µg), Clindamycin (2 µg), Vancomycin (30 µg), and Linezolid (30 µg). Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines. [8]

Detection of Biofilm by Tissue Culture Plate (TCP) Method Biofilm production was assessed using the quantitative Tissue Culture Plate (TCP) method, a reliable and reproducible technique. The procedure involved preparing a 1:100 dilution cell suspension from an overnight bacterial culture in Tryptic Soy Broth (TSB). Subsequently, 200 µl of this diluted cell suspension was transferred in triplicate to wells of a 96-well tissue culture plate. Wells containing only sterile media served as negative controls to ensure that observed adherence was due to bacterial biofilm formation and not artifact. The plates were then incubated at 37°C for 48 hours to allow for biofilm development. After incubation, non-adherent cells were removed by washing the wells three times with Phosphate-Buffered Saline (PBS). The adherent biofilm was then stained with 0.4% crystal violet solution, which binds to the bacterial cells and extracellular matrix of the biofilm. After destaining and drying, the optical density (OD) of the stained biofilm was measured at 570 nm using a microplate reader. Based on the measured OD values at 570 nm, isolates were categorized into three groups for biofilm adherence: non-adherent ($OD \leq 0.12$), weakly adherent ($OD > 0.12$ but ≤ 0.240), and strongly adherent ($OD > 0.240$).[9]

RESULTS

a. Isolation and Distribution of Staphylococci Out of the 130 clinical samples collected and processed, 84 (64.6%) were found to be culture positive. Among these culture-positive samples, 37 (44.4%) were identified as staphylococci. The staphylococcal isolates were further differentiated into *S. aureus* and coagulase-negative staphylococci (CoNS). CoNS represented the predominant group, accounting for 67.5% (n=25) of the total staphylococcal isolates, while *S. aureus* comprised 32.4% (n=12). (Figure 1)

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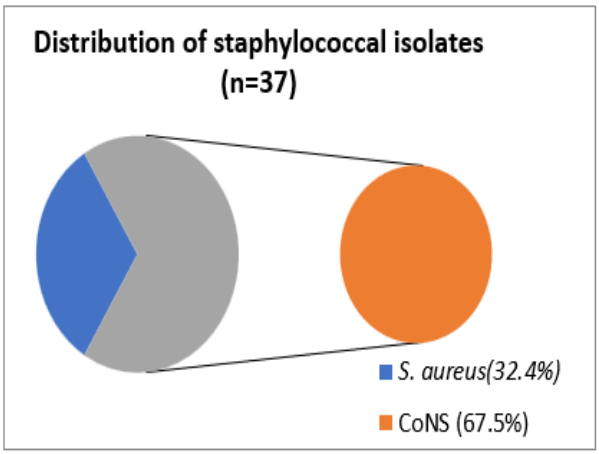

Figure 1: Distribution of *S. aureus* and CoNS

b. Biofilm Production The study revealed a high prevalence of biofilm production among the isolated staphylococci. Overall, 75.6% of the staphylococcal isolates were categorized as strong biofilm producers, and an additional 16.2% were moderate biofilm producers. (Figure 2) CoNS showed a notably higher number of strong biofilm producers compared to *S. aureus*.

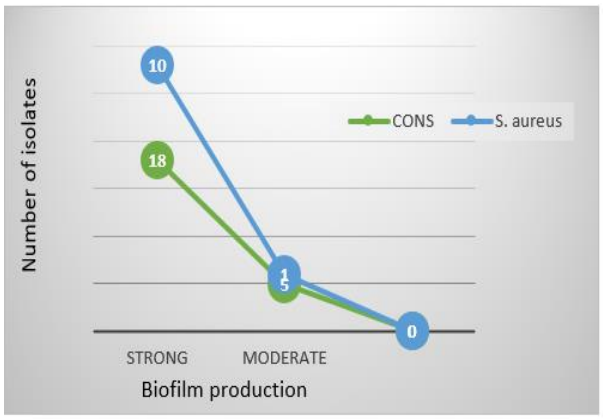

Figure 2

c. Antibiotic Resistance Pattern among Biofilm Producers Antibiotic susceptibility testing revealed significant resistance patterns among the biofilm-producing staphylococci. The study noted a "high resistance" to conventional antibiotics. 91.6% were methicillin resistant Staphylococcus aureus (MRSA) and 84% were methicillin resistant CoNS. However, a crucial finding was that **all isolated strains, regardless of their biofilm-producing capacity, were sensitive to vancomycin and linezolid**. This indicates these antibiotics remain highly effective against the tested isolates. (Figure 3).

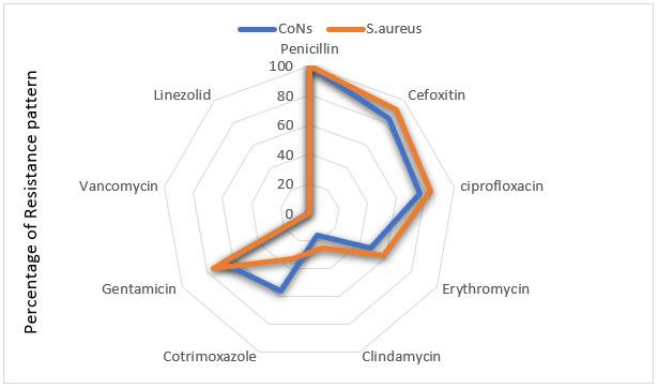

Figure 3: Antibiotic resistance pattern among Staphylococcal biofilm producers

d. Phenotypic Characterization of Coagulase-Negative Staphylococci Speciation of the CoNS isolates provided insight into the prevalence of different species within this group. *Staphylococcus schleiferi* subsp. *schleiferi* was identified as the most common CoNS species, accounting for 20% of the CoNS isolates. This was followed by *S. hemolyticus* (16%) and *S. simulans* (16%). Other species included *S. epidermidis* (12%), *S. xylosus* (12%), *S. lugdunensis* (8%), *S. cohnii* subsp. *cohnii* (8%), *S. caprae* (4%), and *S. hominis* subsp. *hominis* (4%). (Figure 4)

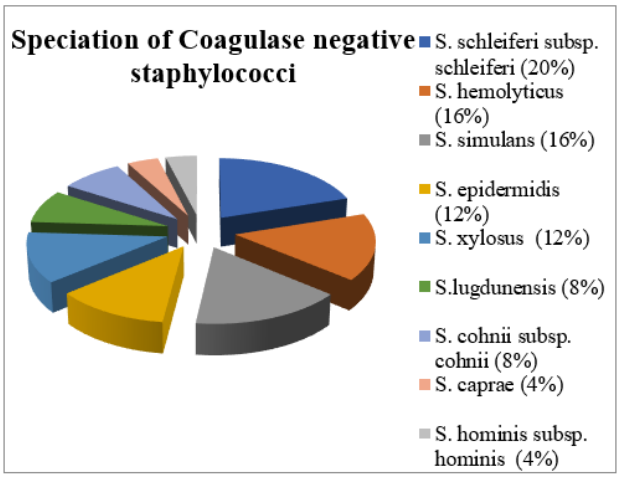

Figure 4: Speciation of Coagulase negative Staphylococci

DISCUSSION

Our study offer crucial insights into the prevalence of staphylococcal infections, their biofilm-producing capabilities, and associated antibiotic resistance patterns in a clinical setting. The isolation of staphylococci from 44.4% of culture-positive samples underscores their significant role as human pathogens. The observed predominance of CoNS (67.5%) over *S. aureus* (32.4%)

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among the isolated staphylococci is particularly noteworthy and reflects a growing trend in clinical microbiology.[10] While CoNS have historically been considered contaminants in microbiological cultures, their increasing isolation as genuine pathogens, particularly in nosocomial settings, highlights their "uniquely qualified" status in modern medical care. This emergence is often linked to the escalating use of invasive medical procedures, a rise in immunocompromised patient populations, and increased survival rates of premature infants, all of whom are highly susceptible to CoNS infections.[7]

Tissue culture plate (TCP) method detected higher biofilm production in the majority of the studies, which correlates in this study as well with 75.6% of staphylococcal isolates demonstrating strong biofilm formation and 16.2% as moderate producers [11-13]. Biofilms provide a protective niche for bacteria, enabling their persistence in chronic infections and contributing significantly to treatment failure. The presence of biofilms renders bacteria less accessible to host immune defenses and significantly reduces the efficacy of antimicrobial agents, necessitating higher drug concentrations that are often unachievable in vivo. This translates into prolonged infections, increased healthcare resource utilization, and poorer patient outcomes.[14] The burden of staphylococcal biofilms on healthcare systems is "tremendous," often requiring surgical intervention to remove infected tissues or implanted medical devices, further emphasizing the severity of these infections. The study's finding that *S. schleiferi* was the most common CoNS species, rather than *S. epidermidis* (which is commonly reported in studies from India and Brazil), suggests potential regional epidemiological shifts or variations in pathogen prevalence. *S. schleiferi* is increasingly recognized for its virulence and ability to cause a variety of infections, making its predominance in this study.[15]

The study's results revealed "high resistance" among biofilm-producing staphylococci to conventional antibiotics. This finding is consistent with other studies globally and locally, which have repeatedly demonstrated a strong association between biofilm formation and increased antimicrobial resistance.[1] The biofilm matrix acts as a physical barrier, limiting antibiotic penetration, while metabolic alterations within the biofilm community can lead to phenotypic resistance even in genetically susceptible strains.[16] Furthermore, genetic elements conferring resistance can be readily transferred within the biofilm community, contributing to the spread of multi-drug resistant strains. Crucially, the study also reported that all isolated strains were sensitive to vancomycin and linezolid. This underscores the continued importance of these antibiotics as last-resort agents for severe staphylococcal infections, particularly those involving multi-drug resistant strains or biofilm production. However, relying on a limited number of effective antibiotics always carries the risk of selecting for resistance.[2] The sustained susceptibility to vancomycin and linezolid provides a window of opportunity for effective treatment, but continuous surveillance for resistance to these vital drugs is imperative to prevent a future therapeutic crisis.[17]

Diagnosing biofilm-associated infections presents unique challenges. Conventional microbiological cultures, which primarily detect planktonic bacteria, may underestimate the true pathogen load or fail to detect slow-growing, biofilm-embedded organisms.[3] Furthermore, the common perception of CoNS as contaminants can lead to misinterpretation of laboratory results, delaying appropriate treatment for genuine infections. The Tissue Culture Plate (TCP) method, as utilized in this study, is a valuable phenotypic screening tool for biofilm production, and its recommendation as a "suitable and reproducible method for screening of biofilm producers in any healthcare setup" is well-founded. However, integrating such methods into routine diagnostics requires resources and expertise. Advanced diagnostic techniques, such as molecular methods (e.g., detection of biofilm-associated genes like *icaA* or *bap*), sonication of implants, or imaging techniques, are often necessary for definitive diagnosis, but these were not within the scope of this phenotypic study.[18]

Prevention and Treatment Strategies Given the complexities of staphylococcal biofilm infections, a multi-approach encompassing robust prevention and effective treatment strategies is essential. Prevention: The study emphasizes that "many of these infections can be prevented or delayed by rigorous application of traditional infection control techniques". This includes fundamental practices such as stringent hand hygiene, meticulous aseptic techniques during medical procedures and device insertions, judicious use of antimicrobial agents to minimize resistance selection, and comprehensive surveillance programs to track infection rates and resistance patterns.[19] Implementing infection prevention bundles for medical devices (e.g., central line bundles, urinary catheter bundles) can significantly reduce the incidence of device-associated biofilm infections. Treatment: The continued sensitivity of all isolates to vancomycin and linezolid in this study is a significant finding for guiding empirical and targeted therapy. However, it is crucial to recognize that antibiotics alone often struggle to eradicate established biofilms. The reduced penetration and altered physiology of biofilm-embedded bacteria necessitate strategies

beyond standard antimicrobial therapy. As highlighted, surgical intervention is often required to remove infected tissues or medical implants, as this physically removes the biofilm reservoir. Research into novel anti-biofilm agents, such as biofilm dispersants, enzymes, bacteriophages, or agents targeting quorum sensing, represents a promising avenue for future therapeutic development, although these are beyond the scope of this study.[20]

LIMITATIONS AND FUTURE DIRECTIONS

This study provides valuable phenotypic data; however, it has certain limitations. The sample size of 37 staphylococcal isolates, while informative, may not fully represent the diversity and prevalence of biofilm-producing staphylococci across all clinical settings. The study is also limited to phenotypic characterization, lacking molecular analysis of biofilm-related genes (e.g., *icaA, bhp, atlE*) or specific antibiotic resistance genes (e.g., *mecA* for cefoxitin resistance) that could provide deeper mechanistic understanding and aid in epidemiological tracking. Furthermore, the single-center nature of the study may limit the generalizability of the findings. Future research should aim for larger, multi-center studies that incorporate molecular techniques for both speciation and the detection of biofilm and antimicrobial resistance genes. Additionally, exploring the efficacy of adjunctive anti-biofilm therapies alongside conventional antibiotics could lead to more effective treatment paradigms.

CONCLUSION

This study comprehensively characterized staphylococcal isolates from diverse clinical samples, elucidating their significant capacity for biofilm production and associated antibiotic resistance profiles. The findings confirm that CoNS are frequently encountered not merely as contaminants but as formidable pathogens in modern healthcare, with their pathogenicity closely linked to biofilm formation. The high prevalence of strong biofilm producers (75.6%) among staphylococci and significant resistance to conventional antibiotics highlight the challenge posed by these infections. The sustained sensitivity to vancomycin and linezolid offers critical therapeutic options, but also emphasizes the need for vigilant surveillance to preserve their efficacy. The Tissue Culture Plate method is a practical and reliable tool for screening biofilm producers and necessitates a renewed commitment to rigorous infection control techniques, coupled with ongoing research into novel diagnostic and therapeutic strategies.

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