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Biofilm production and antibiotic resistance profiles of coagulase-negative *Staphylococci* isolated from hospitalized patients

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ABSTRACT

Coagulase-negative staphylococci (CoNS) have emerged as significant opportunistic pathogens, particularly in hospital settings where they are increasingly implicated in healthcare-associated infections (HAIs). Biofilm formation and colonization on medical devices represents a pivotal determinant in the pathogenesis of CoNS infections, allowing them to adhere to both biotic and abiotic surfaces, evade immune responses and withstand antimicrobial treatments. This study aimed to determine the prevalence of biofilm formation among CoNS and examine its impact on antibiotic resistance using a modified microtiter plate method in clinical samples. Our findings reveal a 100% prevalence of biofilm formation among CoNS isolates, with strong biofilm in 43.33% of strains, moderate biofilm in 20%, and weak biofilm in the remainder. Among the strong biofilm-producing strains, 92.31% exhibited the MRCoNS resistance phenotype and 79.92% showed the cMLSb resistance phenotype. These results illustrate the growing threat posed by biofilm-forming CoNS to modern medicine and highlight the urgent need to develop advanced methods for combating biofilm-producing bacteria.

INTRODUCTION

Coagulase-negative staphylococci (CNS) have emerged as significant opportunistic pathogens, particularly in hospital settings, where they are frequently implicated in healthcare-associated infections (HAIs) [1]. Although traditionally considered as non-pathogenic, CNS species, such as *Staphylococcus epidermidis* and *Staphylcoccus haemolyticus* have demonstrated remarkable adaptability and resilience, often forming biofilms on in-dwelling medical devices such as catheters, prostheses or implants [1-3].

Biofilm formation and colonization on medical devices represents a pivotal determinant in the pathogenesis of CNS infections, allowing them to adhere to both biotic and abiotic surfaces, evade immune responses and withstand antimicrobial treatments [3,4]. Within biofilms, bacteria exhibit characteristics not seen in their planktonic form, including enhanced antibiotic resistance [4,5]. Biofilm-associated bacteria have altered metabolism and gene expression, allowing adaptation to hypoxic environments and nutrient limitations, thereby increasing resistance to antimicrobial therapies [5,6].

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The structural complexity of biofilms, coupled with the phenotypic changes they induce in bacteria, significantly enhances the challenge of treating CNS infections in clinical practice [3]. Moreover, antibiotic resistance among CNS further exacerbates therapeutic options, posing a serious public health concern [1].

In hospitalized patients, CNS-associated infections have become increasingly frequent, particularly among immunocompromised individuals and patients in intensive care units [1,7]. These species are also one of the leading causes of bloodstream infections associated with intravascular catheters and are responsible for about 20% of all infections associated with cardiac devices [1,8].

Moreover, those pathogens may represent antibiotic resistance phenotypes, such as MRCNS (Methicillin-Resistant Coagulase-Negative Staphylococci) and MLSb (Macrolide-Lincosamide-Streptogramine b). MRCNS shows resistance to all β-lactams except the V generation of cephalosporins. It is caused by the acquisition of gene *s*, which encodes a modified Penicillin-Binding Protein (PBP) called PBP2' or PBP2a. This brings about a loss of antibiotic molecule affinity for those proteins. Genes *mecB* and *mecC* are connected with MRCNS as well [9]. MLSb is a phenotype of

resistance to macrolides, lincosamides and streptogramine B, and is connected with methylation of the 23S rRNA, which is the target for these antibiotics. There are two types of MLSb: inducible (iMLSb) and constitutive (cMLSb). The MLSb phenotype is connected with the acquisition of *erm* genes [10].

This study aims to investigate the prevalence of biofilm-producing CNS among hospitalized patients and characterize their antibiotic resistance profiles. In this manuscript, we present findings from a comprehensive analysis of biofilm production and antibiotic resistance in clinical isolates of CNS collected from hospitalized patients.

MATERIALS AND METHODS

Bacterial strains

The study included a total of 30 clinical isolates of CNS collected from blood samples of hospitalized patients with signs and symptoms of invasive infection. The isolates were considered true pathogens and etiologic agents of bacteremia based on the following criteria: at least two separate blood cultures were positive for the same CNS organism, fulfilling the standard diagnostic criteria for diagnosing bacteremia. The microbial strains analysed in this study were derived from routine clinical specimens collected during standard diagnostic procedures. Information regarding the sourced hospital wards is shown in Table 1. The identification of isolates growing in cultures was performed using a biochemical method – the Vitek system (BioMérieux), according to the manufacturer's instructions.

Antimicrobial susceptibility

Susceptibility of staphylococcal isolates to antimicrobial drugs was evaluated via the Vitek system (BioMérieux). The tests were interpreted according to the current recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), Breakpoint tables for interpretation of MICs and zone diameters, Version 13.1, 2023 [11].

Phenotypic characterization of the biofilm-producing ability

To quantitatively determine biofilm production, we employed a modified microtiter plate method as described by Stepanović [12]. Overnight cultures of each bacterial isolate were adjusted with tryptic soy broth (TSB, BioMérieux) to achieve a turbidity equivalent to 0.5 McFarland standard. These suspensions were then incubated overnight at 37°C. Subsequently, the cultures were diluted 1:100 in TSB, and 200 µl aliquots containing 1% glucose were inoculated into four wells of a sterile 96-well flat-bottomed microtiter plate. Negative controls consisting of eight wells containing only TSB with 1% glucose were included. The plates were then incubated overnight at 37°C in ambient air.

After incubation, the contents of the plate were discarded into the medical waste container. 300 µl sterile PBS (previously warmed to room temperature) was added into all wells and removed by flicking the plate. This step was performed three times. After washing, the biofilm was stained by adding 150µl of crystal violet for 15min at room temperature. The plate was rinsed using distilled water and left in the upside-down position at room temperature overnight to dry. After drying, 150 µl of 33% acetic acid was added to each well and allowed to sit for 15 minutes at room temperature. The contents were then transferred to a new plate, and optical density (OD) was measured at 570 nm using a microtiter plate reader BioTek Synergy LX (Agilent). The subsequent calculations were applied so as to interpret the biofilm forming capacity.

A cut-off value (ODc) was established for every plate by way of utilizing the formula: ODc=average OD of negative control + (3× standard deviations of negative control) and interpreted with OD of the strain as follows: a≤ODc (a – mean of OD values of the strain) – no biofilm production, ODc<a≤2xODc – weak biofilm production, 2xODc<a≤4xODc – moderate biofilm production, a>4xODc – strong biofilm production.

Table 1. Characterization of staphylococcal isolates associated with blood infection: phenotypic biofilm production, and antibiotic resistance results

Strain no.	Hospital Ward	Bacterial species	MRCNS, MLSb and LZD resistance profile	Biofilm production capability
1.	Cardiology	*S. epidermidis*	MRCNS, cMLSB	strong
2.	Cardiology	*S. epidermidis*	MRCNS	strong
3.	Nephrology	*S. epidermidis*	MRCNS	strong
4.	Intensive care	*S. epidermidis*	-	weak
5.	Toxicocardiology	*S. epidermidis*	MRCNS, cMLSB	strong
6.	Neurology	*S. epidermidis*	MRCNS	weak
7.	Neurology	*S. epidermidis*	-	moderate
8.	Gynecology and neonatal	*S. epidermidis*	MRCNS, iMLSB	weak
9.	Toxicocardiology	*S. epidermidis*	-	weak
10.	Internal medicine	*S. epidermidis*	MRCNS, cMLSB	weak
11.	Toxicocardiology	*S. epidermidis*	MRCNS, cMLSB	strong
12.	Nephrology	*S. epidermidis*	MRCNS	weak
13.	Loryngology	*S. epidermidis*	MRCNS, cMLSB	strong
14.	Gynecology and neonatal	*S. epidermidis*	-	weak
15.	Internal medicine	*S. haemolyticus*	MRCNS, cMLSB	moderate
16.	Neurology	*S. haemolyticus*	MRCNS, cMLSB	weak
17.	Cardiology	*S. hominis subsp. hominis*	MRCNS, iMLSB	weak
18.	Internal medicine	*S. hominis subsp. hominis*	MRCNS, cMLSB	strong
19.	Oncology	*S. hominis subsp. hominis*	MRCNS, cMLSB	moderate
20.	Oncology	*S. haemolyticus*	MRCNS, cMLSB	strong
21.	Toxicocardiology	*S. hominis subsp. hominis*	MRCNS, cMLSB, LZD	strong
22.	Internal medicine	*S. haemolyticus*	MRCNS, cMLSB	strong
23.	Orthopedic	*S. haemolyticus*	MRCNS, cMLSB	weak
24.	Neurology	*S. warneri*	MRCNS	moderate
25.	Neurology	*S. hominis subsp. hominis*	-	moderate
26.	Nephrology	*S. hominis subsp. hominis*	MRCNS, cMLSB	strong
27.	Neurology	*S. haemolyticus*	MRCNS	weak
28.	Neurology	*S. hominis subsp. hominis*	MRCNS, cMLSB	strong
29.	Internal medicine	*S. hominis subsp. hominis*	MRCNS	moderate
30.	Gastroenterology	*S. hominis subsp. hominis*	-	strong

$cMLS_B$ – constitutive resistance to macrolide-lincosamide-streptogramin B antibiotics; $iMLS_B$ – inductive resistance to macrolide-lincosamide-streptogramin B antibiotics, MRCNS – methicillin resistant coagulase negative staphylococci, LZD – linezolid

RESULTS

Bacterial strains and antibiotic resistance

Clinical strains were identified as *S. epidermidis* (14 strains, 46.67%), *S. hominis* spp. *hominis* (nine strains, 30.00%), *S. haemolyticus* (six strains, 20.00%) and *S. warneri* (one strain, 3.33%) (Table 1).

The study revealed MRCNS, using a cefoxitin screening test, in 24 strains (80.00%). Nineteen strains (63.33%) showed resistance to ciprofloxacin and levofloxacin, while the remaining were classified as susceptible with increased exposure. The iMLSb phenotype was observed in two strains (6.67%), whereas cMLSb was found in 15 strains (50.00%). Resistance to teicoplanin and linezolid was detected in five strains (16.67%) and one strain (3.33%), respectively. Tetracycline resistance was present in nine strains (30.00%), with another nine strains (30.00%) classified as susceptible with increased exposure. Five strains (16.67%) showed rifampicin resistance, and six strains (20.00%) were resistant to trimethoprim/sulfamethoxazole, while seven strains (23.33%) were classified as susceptible with increased exposure. One strain (3.33%) was categorized as susceptible with increased exposure to tigecycline. All strains (100.00%) were susceptible to daptomycin and vancomycin (Table 1).

Phenotypic biofilm production

All isolated strains (100.00%) exhibited biofilm production ability (Table 1, Figure 1). Strong biofilm production was observed in 13 strains (43.33%), including six strains of *S. epidermidis* (46.15%), five strains of *S. hominis* spp. *hominis* (38.46%), and two strains of *S. haemolyticus* (15.38%). Among these strains, 12 out of 13 (92.31%) showed the MRCNS resistance phenotype, 10 strains (79.92%) revealed cMLSb resistance phenotype, nine (69.23%) were resistant to ciprofloxacin and levofloxacin, five (38.46%) to rifampicin, four (30.77%) to tetracycline, three (23.08%) to teicoplanin, one (7.69%) to linezolid and trimethoprim/sulfamethoxazole. Additionally, five strains (38.46%) were categorized as susceptible with increased exposure to tetracycline, and three (23.08%) for trimethoprim/sulfamethoxazole.

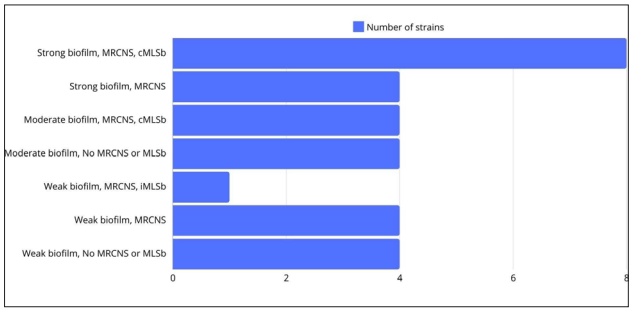

Figure 1. The prevalence of antibiotic resistance phenotypes such as MRCNS and MLSb in relation to the strength of biofilm production

Moderate biofilm production was observed in six strains (20.00%), including three strains (50.00%) of *S. hominis* spp. hominis and one strain (16.67%) each of *S. epidermidis*, *S. warneri* and *S. haemolyticus*. Four out of these strains (66.67%) exhibited the MRCNS phenotype, and two (33.33%) showed cMLSb phenotype. Three strains (50.00%) were resistant to erythromycin, the remaining were susceptible to clindamycin. Additionally, four strains (66.67%) were resistant to ciprofloxacin and levofloxacin, and two (33.33%) were susceptible with increased exposure. There were also several strains resistant to teicoplanin (one strain, 16.67%), tetracycline (two resistant strains, two susceptible with increased exposure), rifampicin (one strain), and trimethoprim/sulfamethoxazole (one resistant strain, three susceptible with increased exposure). One strain (3.33%) exhibited susceptible with increased exposure to tigecycline. There was no resistance observed to linezolid, daptomycin and vancomycin.

Eleven strains exhibited weak biofilm production, including seven strains of *S. epidermidis*, three of *S. haemolyticus*, and one of *S. hominis* spp. *hominis*. Seven strains exhibited the MRCNS phenotype, and five showed cMLSb phenotype. Six strains were resistant to ciprofloxacin and levofloxacin, while the rest were categorized as susceptible with increased exposure. Four strains were resistant to erythromycin with preserved susceptibility to clindamycin, one to teicoplanin, four to tetracycline (two as susceptible with increased exposure), and four to trimethoprim-sulfamethoxazole (one as susceptible with increased exposure). All strains were susceptible to linezolid, daptomycin, vancomycin, tigecycline and rifampicin.

DISCUSSION

Biofilm formation is extremely concerning for modern medicine, as it helps bacteria survive antibiotic therapy and increases the antibiotic concentration required for effective treatment [13]. Currently, researchers report that up to two-thirds of the bacteria responsible for clinical infections can form biofilms [2].

Coagulase-negative staphylococci, including *S. epidermidis*, are responsible for approximately 30% to 40% of all hospital-acquired bloodstream infections. These infections are often directly linked to intravascular catheter infections (ICI), highlighting the significant role of *S. epidermidis* biofilm-forming ability in these cases [3]. Furthermore, CNS are leading causes of prosthetic valve endocarditis (PVE), contributing to 15% to 40% of all PVE incidents, cardiac pacemaker infections with occurrence rates between 0.13% and 19.9% and surgical site infections [14].

Interestingly, multiple studies have shown that in mixed-species biofilm formations, the species involved, despite coexisting in the same biofilm, are spatially segregated and occupy distinct niches. This spatial separation changes the biofilm's observable properties. For instance, Orazi reported that the presence of *S. aureus* and *Pseudomonas aeruginosa* together in mixed-species biofilms leads to increased sensitivity to antibiotics targeting cell walls but also results in resistance to vancomycin [15]. This phenomenon underscores the heightened risk posed by multi-species biofilms and highlights the complexity and difficulty of treating infections associated with these diverse biofilm communities.

Among the staphylococci studied, 100% of the strains tested were able to form a biofilm (30 strains). Of these, 43.33% (13 strains) of the strains were classified as strong biofilm producers, 20% (six strains) were moderate

producers and 36.67% (11 strains) were classified as weak biofilm producers. It should be noted that most of these strains also showed associated antibiotic resistance profiles mainly MRCNS, cMLSb, ciprofloxacin and levofloxacin resistance. This presents an extremely difficult profile to treat clinically.

Biofilm-producing strains often also have an extensive antibiotic resistance profile. Kitti and colleagues report that up to 87.3% of all MRCNS *Staphylococcus* strains isolated in their study were biofilm producers [16]. This is consistent with the result obtained in our study, where MRCNS resistance was found in 80% of the strains studied. Other common resistance groups observed in the strains studied were resistance to ciprofloxacin and levofloxacin (63.33%), as well as to cMLSb (50.00%).

In our study, the most frequently identified strains were *S. epidermidis, S. hominis* spp. *hominis* and *S. haemolyticus*. This trend is consistent with those observed in other works studying this topic, which report that the above-mentioned strains are often responsible for antibiotic-resistant and biofilm-forming infections [17]. This is likely due to their prevalence among the skin microbiota, from which the strain can invade the blood when the skin border is ruptured.

A major medical problem is the formation of biofilms by these strains (especially *S. epidermidis*) on medical devices and biotic surfaces. Some sources suggest that this can lead to the detachment of individual cells, resulting in the spread and colonization of other parts of the body, leading to infections such as endocarditis and septicemia [2].

The presented study demonstrates the great danger posed by the biofilm-forming ability of bacteria and the frequency of its co-occurrence with a variety of resistance types. Currently there are no antimicrobial agents targeting bacteria growing in biofilms, leading to poor treatment results [18]. This leads to a lack of effective tools for biofilm control, despite the widespread prevalence of biofilms. Therefore, it is extremely important to better understand the mechanisms responsible for its formation and to discover methods to effectively combat biofilm-forming strains.

CONCLUSIONS

1. All isolated CNS strains (100%) were capable of biofilm production, which complicates treatment efforts as biofilms enhance bacterial resistance to antibiotics and protect against host immune responses.
2. The study revealed that 80% of all CNS strains exhibited methicillin resistance (MRCNS), indicating a significant challenge in treating infections caused by these pathogens due to their ability to resist commonly used antibiotics.
3. Among the strong biofilm-producing strains, a high percentage showed resistance to multiple antibiotics: 92.31% exhibited the MRCNS resistance phenotype, and 79.92% showed the cMLSb resistance phenotype.

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