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Detection of Biofilm Producing Staphylococcus aureus among different clinical isolates and its antibiotic sensitivity pattern from cancer patients in a tertiary cancer care centre

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Abstract

Background: *Staphylococcus* genus comprising *Staphylococcus aureus* and coagulase negative *Staphylococcus* (CoNS) are widely distributed in nature and can infect diversity of hosts. *Staphylococcus* is the major pathogen causing biofilm associated infections caused by contaminated hospital indwelling devices. **Aim:** 1. To detect and compare the prevalence of biofilm producer and nonproducer *Staphylococcus* isolated from clinical materials by two different methods, viz. tissue culture plate (TCP) method and tube method (TM) 2. Antibiotic sensitivity pattern in all these isolates is observed. **Material and Methods:** A total of 95 clinical *Staphylococcus aureus* isolates were collected from Jan 2021 to Jan 2022. *Staphylococcus* were isolated and identified from various clinical samples by standard microbiological techniques. The in vitro biofilm production was measured using, TM and TCP methods. **Result:** Specimens belonging to age range: 3 - 80 years were collected; M: F – 1: 1.8. Out of 95 isolates studied, 49 were MSSA,44 were MRSA, 2 were CONS. In a total of 95 samples, 35(37%) was found to form biofilm by tissue culture plate method while tube method detected biofilm in 22(23%). 34 % of MRSA and 14% MSSA were biofilm producers. The samples were 100% sensitive to linezolid, vancomycin and Teicoplanin. **Conclusion:** Both methods, TCP and TM showed that *Staphylococcus* isolates have high degree of biofilm-forming ability, the tissue culture method had a higher sensitivity. Surveillance of biofilm formation by *S. aureus* may help in management of infections in cancer patients.

Key Words: Tissue culture plate method, Tube method , Biofilm

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Introduction

Staphylococcus aureus is a clinical pathogen that causes human infections, ranging from mild superficial infections to toxin-associated diseases and severe life-threatening invasive infections. [1]It is widely accepted that *Staphylococcus aureus* is a crucial agent involved in nosocomial infections, which significantly increase morbidity and death among hospitalised patients. This is partly because it can stick to indwelling medical equipment and form biofilm, a multi-layered structure made up of bacterial colonies embedded in the extracellular hydrated polymeric matrix.[2]

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However, they offer an excellent surface for the attachment of adherent bacteria and lead to device-related chronic infections, which will be challenging to cure. Implantable medical devices have become essential in healthcare systems. Many pathogenic bacteria express the ability to build biofilm, an important virulence feature, and *Staphylococcus aureus* are the most frequent etiological agents of device-related infections.[3]

The infections that result are quite diverse, and can include acute infections, such bacteremia and skin abscesses, which are typically brought on by planktonic cells through the generation of secreted toxins and exo-enzymes [4]. Staphylococcus aureus, on the other hand, can attach to and survive on host tissues, such as bone and heart valves, to cause osteomyelitis and endocarditis, respectively, or on implanted objects, such as catheters, prosthetic joints, and pacemakers, in persistent infections.[5,6,7,8]

A biofilm is described as a sessile microbial community in which cells are embedded in a protective extracellular polymeric matrix and connected to a surface or to other cells. This growth mode has different physiologies in terms of gene expression and protein synthesis.[9,10,11]

Initial attachment, biofilm maturation, and dispersal are the three main processes that can be classified as stages in the evolution of biofilms, according to several definitions. An individual planktonic cell initially attaches to a surface by reversibly associating with it; if the cell does not dissociate, it binds irreversibly to the surface. Surface proteins, also known as microbial surface components that recognise sticky matrix molecules, have a role in attachment.

A microcolony develops as a result of cell division and the start of extracellular matrix creation after attachment. A mature biofilm is created as biomass builds up and cell division proceeds. When environmental signals within the biofilm activate the dispersal mechanisms, the cells re-enter a stage of planktonic development and can start new biofilm formation sites.

Staphylococcus aureus biofilm treatment is as follows. Planktonic cells that are vulnerable and metabolically active cells close to the biofilm's surface will perish when exposed to antibiotics. But the biofilm's persister cells and metabolically inactive cells live on and are still shielded from immune responses by the biofilm matrix. Treatment with dispersion agents improves the penetration and clearance abilities of antibiotics. After the biofilm's matrix degrades, antibiotic-sensitive cells are revealed and killed, while the immune system can attack the antibiotic-tolerant cells (such as persisters).[13]

Regulation of biofilm formation in *Staphylococcus aureus*

Many microorganisms commonly grow in the form of biofilms. As a result, exactly like planktonic growth, it is hypothesised that the development of biofilms is regulated by a wide range of processes. About the unique metabolism of biofilms, we know very little. Several regulatory mechanisms have been identified, including the rbf (regulator of biofilm formation), for which this remains enigmatic. [14] Furthermore, according to fairly recent research, the function attributed to the Trap regulator—which is said to affect biofilm formation in response to a peptide termed RIP—is not actually present; [15,16], rather, it is the result of a second site mutation, most likely in the agr system.[17,18,19]

The leading cause of morbidity and mortality in cancer patients is still infections. Staphylococcus aureus attaching to surfaces of different materials and forming a biofilm are the first steps in an infection, according to research. The action of antibiotics is hampered by biofilms because they make the cells less accessible to the host's defensive mechanism. Thus, techniques for detecting strains with a propensity for biofilm development are required to create efficient biofilm control strategies and enhance patient care.[20]

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The present study aims in detecting the prevalence of biofilm forming capability of *Staphylococcus aureus (*MRSA, MSSA &CONS) in cancer patients, and to study the antibiotic sensitivity pattern of these isolates

Aims & Objectives

1. To detect the prevalence of biofilm producer and nonproducer *Staphylococcus aureus* isolated from clinical materials in our laboratory by two different methods, viz. tissue culture plate (TCP) method and tube method (TM)
2. To compare the above-mentioned different methods for biofilm production.
3. Antibiotic sensitivity pattern in all these isolates

Materials And Methods

The present study was a retrospective study and done.

This is a cross- sectional study done at Department of Microbiology, Kidwai memorial institute of oncology, Bangalore for a period of 12 months, between Jan 2021 to Jan 2022

Inclusion criteria– Cancer Patients belonging to all age groups

Exclusion criteria– Patients without cancer

Bacterial strains

A total of 95 *Staphylococcus aureus* isolates from clinical samples such as pus, blood, throat swabs and were obtained from. The isolates were confirmed as *Staphylococcus aureus* by standard microbiological techniques including Coagulase and Catalase.[21] The cultures were inoculated in nutrient agar deeps and preserved at -20ºC. Standard *S. aureus* ATCC 25923 (strong biofilm producer), *S. aureus* ATCC 20372 (moderate biofilm producer), and *S. aureus* ATCC 12228 (non-biofilm producer) were included in the study as a reference strains.

Detection of biofilm formation

Tube Method (TM)

Tube method (TM) is a qualitative assay for detection of biofilm producer microorganism, as a result of the occurrence of visible film.

Procedure

In this method candida isolates are inoculated in 10 ml of Brain Heart Infusion Broth (BHIB) with 2% sucrose and incubated at 37°C for 18 – 25 hours. The tubes are then decanted and washed with phosphate buffer saline (PBS pH 7.2). tubes are dried and Stained with crystal violet (0.1% w/v) for half an hour. Excess stain was removed; tubes were then dried. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Tubes were examined, and the amount of biofilm formation was scored as absent, moderate or strong.[19]

Microtiter Plate Assay

Microtiter plate (MtP) assay is a quantitative method to determine biofilm production by microplate reader.

Procedure

Isolates from fresh agar plates were inoculated in brain heart infusion broth (BHIB) with 2% sucrose. It was the incubated at 37°C for 18–24 hours in a stationary condition. The broth with visible turbidity was diluted to 1 in 100 with fresh medium. Individual wells of flat bottom polystyrene plates were filled with 0.2 ml of the diluted cultures, and only broth served as a control to check sterility and nonspecific binding of the medium. These plates were incubated at 37°C for 24 hours. After incubation, the content of the well was removed and were washed 4 times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating “planktonic” bacteria. Biofilms formed by adherent “sessile” organisms in plate

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were fixed with 2% sodium acetate for half an hour and stained with 0.1% w/v crystal violet for another half hour. Excess stain was rinsed off by washing with deionized water and plates were kept for drying. Adherent bacterial cells usually formed a biofilm on all side wells and were uniformly stained with crystal violet. Optical densities (OD) of stained adherent bacteria were determined with a micro Enzyme-Linked Immunosorbent Assay auto reader at wavelength of 570 nm (OD 570 nm) and were graded as per Christensen *et al*.

MEAN OPTICAL DENSITIES VALUE	**BIOFILM FORMATION**
<O.120	NONE / WEAK
0.120 – 0.240	MODERATE
≥0.240	HIGH

Antimicrobial susceptibility test

Antimicrobial susceptibility tests of the clinical isolates against different antimicrobials were performed in Müller– Hinton agar (MHA) using the standard disk diffusion technique (modified Kirby–Bauer method) and interpreted as per Clinical and Laboratory Standards Institute guidelines.[23] The following antimicrobial agents were tested: ampicillin (10 µg), cefoxitin (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), clindamycin (2 µg), cotrimoxazole (25 µg), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), minocycline (30 µg), rifampicin (5 µg), teicoplanin (30 µg), tetracycline (30 µg) and vancomycin (30 µg) (HiMedia Laboratories, Mumbai, Maharashtra, India). S. aureus ATCC 25923 was used as the control organism. Isolates were considered multidrug resistant (MDR) based on the guidelines recommended by the joint initiative of the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC). According to those guidelines, the isolates showing non-susceptibility to at least one agent in three or more antimicrobial categories were identified as MDR.[24]

Screening of MRSA

All the isolates identified as *S. aureus* were further screened for methicillin resistance using the cefoxitin disk. Test inoculum (0.5 McFarland standards) was inoculated onto MHA by lawn culture. Cefoxitin disk (30 µg) was placed on the agar plate and incubated overnight at 37 °C. On the following day, the zones of inhibition were measured, and those ≤21 mm in diameter were considered to be MRSA.[24] *S. aureus* ATCC 25923 and ATCC 43300 were used as negative and positive controls, respectively.

Statistical analysis

The Statistical software namely SPSS 22.0, and R environment ver.3.2.2 were used for the analysis of the data and Microsoft word and Excel used to generate graphs, tables etc. The demographic data were analysed using descriptive statistics, and TCP AND TM method were compared using independent t-test. Chi-square test was used for analysis of categorical data. A *P-value* of <0.05 was considered statistically significant.

Results

Age range was from 3-80yrs with 62 females (59%) and 33(41%) males and female: male ratio 1.8: 1(Table /Fig1). Pus was the most common type of sample sent for culture and sensitivity (Table /Fig2).

The different *Staphylococcus aureus* distribution based on antibiotic sensitivity pattern was as follows,49 were MSSA,44 were MRSA, 2 were CONS. Among the *Staphylococcus aureus* isolates,35(37%) was found to form biofilm by tissue culture plate method while tube method

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detected biofilm in 22(23%) (Table /Fig3,4,5). Tissue culture method was most sensitive for detection of biofilm production. P VALUE:<0.02742 and was statistically significant. MRSA produced 34 % of biofilm when compared to MSSA which was 14% by tube method and 47% and 28% by tissue culture method respectively (Table /Fig6,7).19 and 2 cases of MRSA ,5 and 9 cases of MSSA had moderate and high degree of positivity by tissue culture plate method (Table /Fig8). The sensitive pattern of following antibiotic was as shown Table /Fig9. The samples were 100%sensitive to linezolid, vancomycin and Teicoplanin.

Table I: Age and sex wise distribution

AGE RANGE	Female	(%)	Male	(%)
1-10	3	4.84%	2	6.06%
11-20	1	1.61%	3	9.09%
21-30	2	3.23%	2	6.06%
31-40	10	16.13%	2	6.06%
41-50	18	29.03%	8	24.24%
51-60	7	11.29%	7	21.21%
61-70	14	22.58%	5	15.15%
71-80	7	11.29%	4	12.12%
Total	**62**	**100.00%**	**33**	**100.00%**

Table II: Biofilm production by tissue culture plate method and tube adherence method

	Tissue culture plate method	Tube adherence method
Biofilm positive, n (%)	35(37%)	22 (23%)
Biofilm negative, n (%)	60 (63%)	73 (77%)
Total	95	95

Table III: Biofilm production in various species of Staphylococcus aureus.

	Tube method		Tissue culture plate method	
	positive	negative	positive	Negative
CONS	0	2	0	2
MRSA	15	29	21	23
MSSA	7	42	14	35

Table IV: Tissue culture plate method: degree of positivity.

Tissue culture plate method (degree of positivity)	HIGH	MOD
MRSA	2	19
MSSA	5	9

Table V: Antibiotic sensitivity pattern

Antibiotic name	%of isolate sensitive
Ciprofloxacin	22%
Levofloxacin	68%
Co-trimoxazole	60%
Erythromycin	60%
Gentamycin	87%

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Linezolid	100%
Penicillin	8%
Teicoplanin	100%
Vancomycin	100%

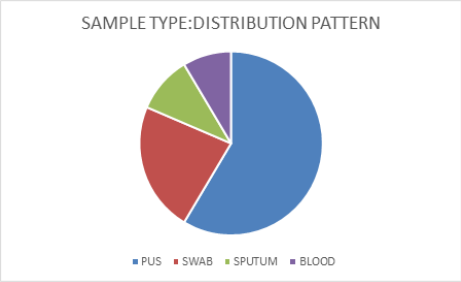

Figure 1: Sample Type Analysed

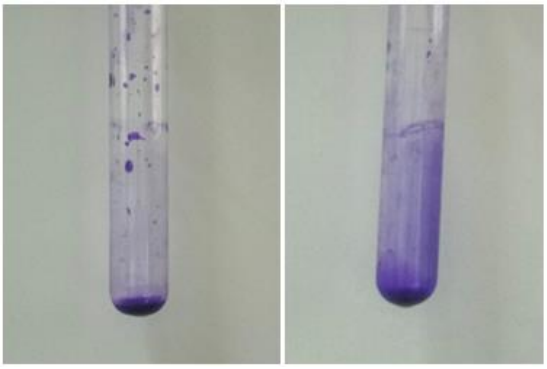

Figure 2: Tube adherence method: negative and positive results

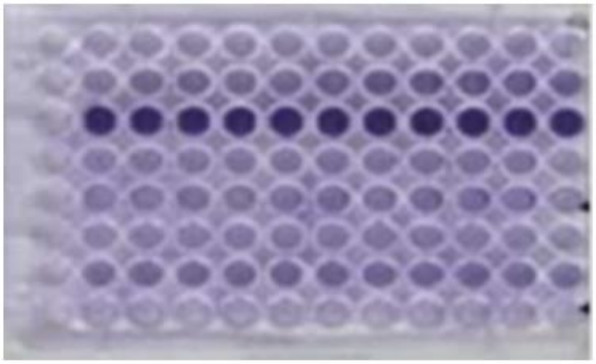

Figure 3: Microtiter plate assay indicating biofilm production

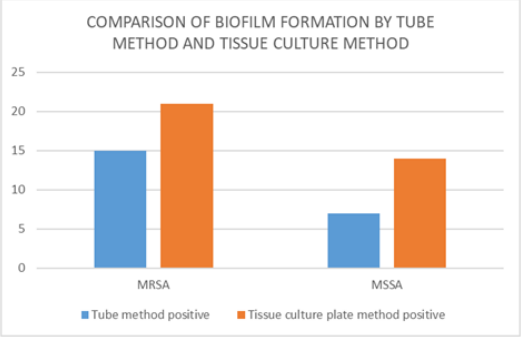

Figure 4: Comparison of biofilm formation in MRSA and MSSA by tube adherence method and tissue culture plate method

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Discussion

The age group with the highest number of isolates was in a range of 60-70 yrs. Wound infection had the highest frequency of *S. aureus* isolates (30.7%) in the study. Males (62.0%) were more infected than females (38.0%).[25] our study had commonest age group between (41-50) yrs. with females 59% and males 41%.

In our study, we studied 95 samples exclusively and compared two methods of biofilm detection that can be used in routine clinical laboratories. We evaluated 95 isolates by two methods (that can be used in routine clinical laboratories) for their ability to form biofilms. Out of the 95 isolates, the TCP method detected biofilm in 35 (37%) when compared to TM which detected biofilm in 22(23%) isolates only. The TCP was found to be most sensitive than TM. Tissue culture method was most sensitive for detection of biofilm production. *P value*:<0.02742 and was statistically significant.

In a study on 110 isolates by Hassan et al, [26] reported that the TCP method detected biofilm in 70 isolates (63.6%), TM in 54 (49%) In another study by Mathur et al,[27]out of the total 152 isolates tested for biofilm formation, 47.3%, 41.4% isolates were biofilm producers as detected by TCP, TM. In a study done by Panda et al, out of the 300 isolates, the TCP method detected biofilm in 137 isolates (45.6%), TM detected biofilm in 118 isolates (39.3%) [25]

Comparison of the grading of the biofilm detected by different methods in various studies is shown in Panda PS et al.[25]

Our study results correlated well with these study results. All the studies suggested that the though TM correlated well with the TCP method for strong biofilm detection, it was difficult to discriminate moderate and weak/none biofilm production by TM. This difference could be attributed to subjective observer's assessment used in TM as compared to the more accurate objective assessment in TCP**.**

The sensitivity pattern of *S.aureus* to the following antibiotics; Gentamicin, Amoxycillin/clavulanate, Streptomycin, Cloxacillin, Erythromycin, Chloramphenicol, Cotrimoxazole, Tetracycline, Penicillin, Ciprofloxacin, Ofloxacin, Levofloxacin, Ceftriaxone, Amoxycillin and vancomycin were 92.4%, 63.0%, 44.2%, 35.8%, 52.4%, 61.9%, 15.5%, 31.2%, 7.1%, 78.9%, 76.6%, 100%, 71.4%, 30.7% and 100% respectively. Methicillin resistant isolates were sensitive to Levofloxacin 93.7% and Ofloxacin 68.7%. [28]. Our study showed 100% sensitivity to linezolid, teicoplanin and vancomycin.

In a study done by Bhat N et al, 140 patients had positive cultures, representing 272 specimens and 306 isolates. Common specimens sent for culture were blood sputum, urine, and pus. 13.72% infections were caused by *Staphylococcus aureus* ,50% of the *Staphylococcus aureus* spp. were methicillin resistant, but all were sensitive to vancomycin[29] .

According to a study Silva et al , all MRSA strains were capable of adhering to the microplate and form biofilms. Understanding the ability of MRSA strains from different types of infections to form biofilms is the first step towards a possible solution for biofilm-related infections.[30]

Our study had 95 cancer patients with *Staphylococcus aureus* growth with pus being the most common sample. MRSA were more biofilm producers when compared to other two species.

Conclusions

37% of *Staphylococcus aureus* isolates obtained from cancer patients were biofilm producers. Both methods, TCP and TM showed that *S. aureus* isolates have high degree of biofilm-forming ability, the tissue culture method had a higher sensitivity. Surveillance of biofilm formation by *S. aureus* may help in management of infections in cancer patients which will help in early treatment and prevent emergence of multidrug resistant strains.

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Higher rate of antimicrobial resistance is demonstrated by biofilm producers than by biofilm non-producers. The biofilm-positive strains have a higher tendency to exhibit multidrug resistance and methicillin resistance compared to biofilm-negative strains. This may lead to the high risk of impairment in the wound healing and dissemination of the infection enhancing morbidity and mortality of the admitted patients. Therefore, we recommend regular surveillance of biofilm formation in *S. aureus* wound isolates and their antimicrobial resistance profiles. This may help us to formulate an effective antimicrobial policy for the early treatment of wound infection.

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