VOLUME - 12, ISSUE - 08, AUGUST - 2023 • PRINT ISSN No. 2277 - 8160 • DOI : 10.36106/gjra

Original Research Paper

Medical Microbiology

BIO FILM PRODUCING CLINICAL STAPHYLOCOCCUS AUREUS ISOLATES AUGMENTED PREVALENCE OF ANTIBIOTIC RESISTANT CASES IN A TERTIARY CARE HOSPITAL

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ABSTRACT Bio film-mediated infections in the hospital environment often led to increase in morbidity and mortality posing a high socio-economic burden, especially in developing countries. Since biofilm formation and antibiotic resistance function dependent on each other, biofilm detection in clinical isolates would be advantageous in treatment decision. In this premise, this study aimed to investigate the biofilm formation and its association with antibiotic resistance in clinical*Staphylococcus aureus* isolates from hospitalized patients visiting a tertiary care hospital. Bacterial cells isolated from clinical samples identified as *Staphylococcus aureus* were examined for in-vitro biofilm production using Tube method (TM) and Congo red agar (CRA) method. Among 90 *Staphylococcus aureus* isolates, as indicated by Cefoxitin disk diffusion assay, 61 (67.8%) isolates were identified as MRSA and the remaining 29 (32.2%) isolates as MSSA strains. TheMRSA isolates were significantly more resistant to majority of the antibiotics than the MSSA strains. A majority of MRSA strains (20%) showed inducible clindamycin resistance. Detection of in vitro production of biofilm revealed the association of biofilm with methicillin as well as inducible clindamycin resistance among the clinical *S. aureus* isolates. Biofilmproduction was higher in MRSA strains as compared to the MSSA strains. The Strong biofilm indicated increased possibility ofantibiotic resistance or tolerance that is likely to lead to treatmentfailures in MRSA infections.Implementation of early detection strategies will help to identify bio film producing *S. aureus* cases to prevent occurrence of treatment failures of *staphylococcal infections* in a tertiary care hospital.

KEYWORDS : Staphylococcus aureus, MRSA,MSSA, Inducible clindamycin resistance,Bio film,Tube method (TM), Congo red agar (CRA) method

INTRODUCTION

Staphylococcus aureus, a notorious human pathogen, is a major cause of the community as well as healthcare associated infections.[1] It can cause a diversity of recalcitrant infections mainly due to the acquisition of resistance to multiple drugs, its diverse range of virulence factors, and the ability to produce Bio film in indwelling medical devices. Such bio film associated chronic infections often lead to increase in morbidity and mortality posing a high socio-economic burden, especially in developing countries. Bio films are the aggregation of bacteria embedded in a self-produced extracellular matrix of exopolysaccharides (EPSs), proteins and some micro molecules such as DNA. They can form on both biotic and abiotic surfaces. Since bio film formation and antibiotic resistance function dependent on each other, detection of bio film expression in clinical isolates would be advantageous in treatment decision. Bacteria within the bio film are not only protected from the host immune systems but also from the antimicrobial agents contributing to treatment failures and recurrent infections. There has been significant interest in assessing the possible relationship between the multidrug-resistant (MDR) status and the bio film-producer phenotype in bacteria.[2,3] Effective measures to eradicate bio film harbouring bacterial cells in-vivo conditions are still poorly identified.

This highlights the importance of understanding the mechanism of bio film formation and its resistance to antimicrobial substances for a successful treatment.[4]In a resource limited country like India, early detection of bio film formation in clinical isolates could be essentially an important practice inprevention and management of nosocomial infections. In this premise, we attempt to investigate the association of bio film formation with resistance to various clinically relevant drugs as well as inducible clindamycin resistance (ICR) using standard microbiological techniques and D-test, respectively in the clinical *S. aureus* isolates received in a tertiary care hospital.

MATERIALS AND METHODS

The Cross-sectional study was conducted at Department of Microbiology after approval by the Institutional Scientific and Ethics Committee for the period of 6 months.

Sample Collection and Processing

Collection and Identification of Isolates

Clinical samples including Blood, Pus, Urine, Sputum, Medical devices like CVC (central venouscatheter), Tracheostomy tube and Tissues were received in thelaboratory. From all clinical samples processed during study period, *Staphylococcusaureus* isolates wereidentified on the basis of colony morphology on Nutrient agar, Blood Agar and Mannitol Salt Agar, Gram stain, and different biochemical tests. The yellow coloured, moist, round, glistening opaque colonies with ß or weak haemolysis on blood agar showing typical *staphylococcal* bunch were subjected to a series of biochemical tests. The isolates exhibiting positive test result to catalase, slide and tube coagulase, Methyl red, Voges Proskauer, Nitrate Reduction, Lactose, Mannitol, Maltose, Mannose, Sucrose and Trehalose fermenting was confirmed as *Staphylococcusaureus*.

Antibiotic sensitivity test

Antibiotic sensitivity test was done on Muller-Hinton agar (MHA) using following antibioticdiscs-Penicillin(10units), Ampicillin(10µg),Ciprofloxacin(5µg),Cefotaxime(30Âµg), Erythromycin(15Âµg), Co-trimoxazole(25Âµg), Amikacin (30Âµg), Gentamicin(10Âµg), Linezolid(30Âµg), Vancomycin (30Âµg), Antibiotics discs were procured from HI Media Laboratories Pvt. Ltd, India. ATCC *Staphylococcus aureus*

25922 was used as control. Antibiotic sensitivity test was done as per Kirby-Bauer disc diffusion method.[5]

Resistance Detection of Clinical *Staphylococcus aureus* isolates

Methicillin-resistance

Methicillin-resistance was detected by placing Cefoxitin (FOX) disks in Mannitol salt agar (MSA) plates. Zone diameters less than 22 mm were considered as positive for Methicillin-resistance. MSSA *S. aureus* ATCC 29213 and MRSA *S. aureus* ATCC 43300 were used as quality control strains.

Screening of Inducible Clindamycin Resistance

The double disk diffusion or D-zone test as outlined in CLSIdocument M100-S24 (CLSI, 2015) was performed to examine whether the Erythromycin resistant isolates expressed inducible Clindamycin resistance.[6] Briefly, the bacterial cells from the *S. aureus* isolates were diluted to 0.5 McFarland standard and spread over the Mueller Hinton agar (MHA) plate, on which Erythromycin (15 μg) disk and Clindamycin (2 μg) disk was placed 15–26 mm edge to edge apart. The plates were incubated at 35°C for 16–18 h in aerobic condition. Flattening of the zone of inhibition of clindamycin adjacent to the Erythromycin disk was regarded as D-test positive (Figure 1).

Figure 1. D-test showing inducible clindamycin resistance

Detection of Bio film-production

Bio film-production was assessed using Tube-adherence Method (TM) and Congo red agar (CRA) plate method.

1. Tube adherence Method

10 ml Trypticase soy broth with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar individually.

Broths were incubated at 37°C for 24 hours. The cultures were decanted and tubes were washed with phosphate buffer saline (pH 7.3). The tubes were dried and stained with 0.1% crystal violet. Excess stain was washed with deionised water. Tubes were dried in inverted position. In positive bio film formation, a visible stained film was seen lining the wall and bottom of the tube. Experiments were done in triplicate for 3 times and read as absent, weak, moderate and strong(Figure2).[7,8]

2. Congo red agar method

The medium composed of Brain heart infusion broth (37 gm/l), sucrose (5 gm/l), agar number 1 (10 gm/l) and Congo red dye (0.8 gm/l). Congo red stain was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes. Then it was added to autoclaved Brain heart infusion agar with sucrose at 55°C. Plates were inoculated with test organism and incubated at 37°C for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicated bio film production (Figure3).[9]

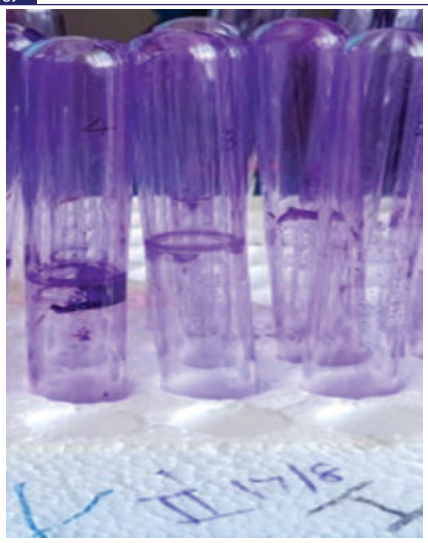

Figure 2. Biofilm production by tube method.Tube 1: +++, Tube 2: ++, Tube 3: +, Tube 4: blank

Figure 3. Biofilm producing isolates with black colonies on Congo Red Agar medium

Statistical analysis

Data from this study was analysed descriptively using SPSS 22.0 software. Chi-square test was used to compare between groups of clinical isolates and P-values < 0.05 was considered statistically significant.

RESULTS

Among 90 *Staphylococcus aureus* isolates, as indicated by Cefoxitin disk diffusion assay, 61 (67.8%) isolates were identified as MRSA and the remaining 29 (32.2%) isolates as MSSA strains. TheMRSA isolates were significantly more resistant to majority of the antibiotics than the MSSA strains (Table 1). However, among all antibiotics, Linezolid, Vancomycin and Tetracycline were found to be the most effective against MRSA strains.

Table1. Antimicrobial Susceptibility Test (AST) results of MRSA and MSSA strains

Antibiotics	MRSA(n=61)		MSSA(n=29)		Total(n=90)	
Cefoxitin	61	67.8%	0	0%	61	67.8%
Penicillin	59	65.6%	1	1.1%	60	66.7%
Clindamycin	6	16.7%	4	4.4%	10	21.1%
Erythromycin	49	54.4%	23	25.6%	72	80 %
Ciprofloxacin	30	33.3%	2	2.2%	32	35.5%
Gentamicin	27	30%	4	4.4%	31	34.4%
Tetracycline	5	5.6%	1	1.1%	6	6.7%

Inducible Clindamycin Resistance

Among 90 *S. aureus* isolates, 72(78.9%) were resistant to Erythromycin. When these isolates were subjected to D-test, 10 (11.1%) isolates showed resistant to both erythromycin

andclindamycin indicating constitutive MLSB phenotype. Out of 80 isolates that were sensitive to clindamycin, 35 (38.9%) alsoshowed positive D-test indicating inducible MLSB phenotype, whereas 27 (30%) showed true sensitivity to Clindamycinas they were D-test negative indicating macrolide sensitive (MS) phenotype. The susceptible phenotype (E-S, CD-S) wasexhibited by 18 (20%) of isolates (Table 2). Among MRSA, theconstitutive MLSB and inducible MLSB phenotype was 6 (6.7%) and 18 (20%) respectively, while in MSSA, the constitutiveMLSB phenotype was 4(4.4%) and inducible MLSB phenotype was 147(18.9%). High prevalence of ICR in MRSA cases indicate the importance of implementation of D-test in regularlaboratory diagnostics to minimize the risk of treatment failures due to this phenomenon.

Table -2 Prevalence of Inducible Clindamycin Resistance (ICR) in MRSA andMSSA strains

Phenotype	MRSA		MSSA		Total		P value
	n	%	n	%	n	%	
E-S, CD-S	12	6	6	6.7	18	20	<0.001
E-R, CD-R (Constitutive MLSB)	6	6.7	4	4.4	10	11.1	
E-R, CD-S (Inducible MLSB-D Test +ve)	18	20	17	18.9	35	38.9	
E-R, CD-S MS Phenotype (D Test -ve)	25	27.8	2	2.2	27	30	
Total	61	67.8	29	32.2	90	100	

Biofilm Production

Tube method (TM) was used to examinethe thickness of biofilm. The result demonstrated that biofilmproduction was higher in MRSA strains as compared to the MSSA strains not only quantitatively but also qualitatively. Strongbiofilm indicated increased possibility ofantibiotic resistance or tolerance that is likely to lead to treatmentfailures in MRSA infections (Table 3). In-vitro detection of biofilm production by MRSA and MSSA strains by commonly used phenotypic assays (CRA, TM) was depicted in Table-4.

Table3. Biofilm production among *Staphylococcal* isolates by tube method

Biofilm production	MRSA		MSSA		Total		P value
Strong (+++)	12	13.3%	0	0%	12	13.3%	<0.001
Moderate (++)	15	16.7%	4	4.4%	19	21.1%	
Weak (+/-)	34	37.8%	25	27.8%	59	65.6%	

Table4. *In-vitro* detection of biofilm production by MRSA and MSSA strains by commonly used phenotypic assays (CRA, TM)

Method	Biofilm production	MRSA	MSSA	Total	P value
TM	Positive	27(30%)	4(4.4%)	31 (34.4%)	< 0.001
	Negative	34(37.8%)	25(27.8%)	59 (65.6%)	
CRM	Positive	1(1.1%)	0(0%)	1(1.1%)	
	Negative	60(66.7%)	29(32.2%)	89 (98.9%)	

DISCUSSION

Bio films are a group of micro-organisms that attached to a surface and covered by an exopolysaccharide matrix. Bacterial bio film has long been considered as avirulence factor contributing to infectionassociated with various medical devices and causing Nosocomial infection.[10,11] The exact process by which bio film producingorganisms cause disease is poorly understood. However, suggested mechanisms are Detachment of cells from medicaldevice bio film causing bloodstreamor urinary tract infection, Endotoxinformation, Resistance to host immune system and Generation of resistance through plasmid exchange.[12]

In this study,90*Staphylococcal* spp. were isolated fromvarious clinical samples namelyBlood, Pus, Urine, Sputum, Medical devices like CVC (central venouscatheter), Tracheostomy tube and Tissuesetc. Out of 90 *Staphylococcal* isolates, 61 (67.8%) isolates were identified as MRSA and the remaining 29 (32.2%) isolates as MSSA strains. This result shows higher cases ofMRSA in comparison to the previous studies which reportedonly 19 to 45.9% of MRSA cases in clinical samples.[13,14] The MRSA isolates were significantly more resistant to majority of the antibiotics than the MSSA strains which wasin accordance with previous observations by Ansari et al. study highlighted the risk factors contributing to the increasing rateof resistance mainly include lack of regulation of antibiotics availabilityeven without prescription and prescription by unauthorizedpersonnel, self-medication, pharmacies promoting their productsthrough clinicians and lack of laboratory facilities to detectthe antibiotic resistance among others.[15] However, among all antibiotics, Linezolid, Vancomycin and Tetracycline were found to be the most effective against MRSA strains. We detected inducible clindamycin resistance in 80% ofisolates, which is significantly higher than previous studies thatreported only in 12.4 to 22.4% of cases in Ansari et al. study.(15) Inthe present study,the MS phenotype and constitutive MLSB phenotype was higheramong MRSA (27.8 and 20%) when compared with MSSA (2.2and 18.9%) which in contrast to Sasirekha et al study showed higherMLSB phenotypes in MSSA as compared to the MRSA strains.[16] The higher incidence of MLSB in this study indicates the importance of D-test in routine laboratory diagnostics for preliminary identification of ICR which would be implemented for effective clinical prescript ion minimizing the treatment failures.

All isolates were tested by two in vitro screening tests for bio film production namely Tube Method and Congo Red methods. Strong bio film formation as measured qualitatively was significantly higher in MRSA strains as compared to the MSSA strains. The significant andclinically relevant observation was that the highresistance shown by biofilm producers toconventional antibiotics than non-biofilmproducers. This observation was supported by other studies also.[12,17] This study showed that TM is the better screening testfor biofilm production than CRA. Thetest is easy to perform and assess bothqualitatively and quantitatively.

CONCLUSION

This study gave information about the status of bio film producing clinical *S. aureus* strains and their association with multiple antibiotic resistances, highlights the importance of early detection strategies in routine diagnostics. Implementation of early detection strategies will help to identify bio film producing *S. aureus* cases to prevent occurrence of treatment failures of *staphylococcal infections* in a tertiary care hospital.

Acknowledgments

We are thankful to the Dean and staff members of Microbiology Tirunelveli Medical College for their contribution during laboratory investigation and data collection.

Conflict Of Interest

The authors declare that there is no conflict of interest.

Authors' Contribution

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Funding
None.

Data Availability
All datasets generated or analyzed during this study are included in the manuscript.

Ethics Statement
This study was approved by the Institutional Ethics Committee, Tirunelveli Medical College, Tirunelveli, Tamil Nadu.

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