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Original Research Article

Biofilm production and its effects on virulence of MRSA: a study in tertiary care hospital, Delhi

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ABSTRACT

Background: The aim of this study was to study the prevalence of biofilm formation in MRSA and its effect on virulence and the antimicrobial resistance pattern on MRSA strains from different clinical samples.
Methods: A total of 221 isolates of *S. aureus* isolates were selected from various clinical specimens. Prevalence was estimated according to age, sex, and location. The antibiotic susceptibility test was conducted according to the guidelines of CLSI by the VITEK 2 automated system. 113 strains were identified as MRSA by cefoxitin disc methods which were then subjected to Microtiter plate assay method to confirm phenotypic biofilm formation.
Results: 51.13% isolates were resistant to methicillin, and 48.86% isolates were methicillin sensitive. The most common source of MRSA isolation was blood. MRSA isolates were mostly isolated from male. 33.63% MRSA and 19.44% MSSA isolates were strong biofilm producers while 12.38% MRSA and 14.81% were low biofilm producers. The resistance for commonly used antibiotics like benzyle penicillin, ciprofloxacin, cotrimoxazole, and erythromycin was more in MRSA strains and MIC was higher in biofilm producers.
Conclusion: Statistical difference was observed between MSSA and MRSA regarding biofilm formation and antimicrobial resistance. A Biofilm producer shows resistance to many antibiotics and also make host immunity in effective. In hospitals Biofilm production should be checked regularly before giving treatment. And research should be done to find out other effective drugs to eradicate biofilms.

Keywords: Biofilm, Methicillin-resistant *Staphylococcus aureus*, Biofilm producers

INTRODUCTION

Staphylococcus aureus is regarded as one of the most pathogenic species of the genera Staphylococcus. Problem arises with its carriage. It is a part of commensal flora found in the anterior nares, axillae and moist areas. Adapted to colonize our bodies which in turn probably provide big ecological niches for these bacteria. All of us daily counter the bacteria, but few people remain carriers over longer periods of time. Carriers are asymptomatic, bacteria is not harmful and could even be protective for a host if infected by *S. aureus*. Three types of carriers have been described: some who always carry them, few who carry the organism intermittently with different strains, and few people who never have *S aureus.* Children are persistent carriage than adults. Persistent carriers have high risk of infection and intermittent and non-carriers shows low risk. [1]

S. aureus is reported to have ample potential to cause human infections, presenting as mild to severe skin infections to life threatening ones such as osteomyelitis, endocarditis, and pneumonia. It is the second most common cause of hospital-acquired bloodstream infections. Patients undergoing surgery acquire at least one nosocomial S. aureus infection in 20% cases,

resulting in increased morbidity, mortality, hospital stay, and costs. [2-3]

S. aureus has found to be highly expert at developing resistance during the antibiotic era. Penicillin resistant *S. aureus* strains were emerged in hospitals in the mid-1940s, soon after the introduction of penicillin,[4-5] Strains were found to producing an enzyme penicillinase which hydrolysed the β-lactam ring of penicillin. Within a decade penicillin-resistant *S. aureus* strains became pandemic [6]. A new β-lactam antibiotic, methicillin was introduced and within few years the first case of methicillin-resistant *S. aureus* (MRSA) was documented. Methicillin-resistance confers resistance to all β-lactam antibiotics (penicillin, cephalosporins and carbapenems). Methicillin-resistant *S. aureus* (MRSA) strains resist other group of antibiotics along with beta lactams after being in phase of development of antibiotic resistance becoming more virulent, for last four decades. [7]

To enhance the problem, S. aureus can live in the biofilm state. Biofilms are colonies of bacteria covered in a self-produced extracellular polymeric matrix that gets attached to biotic and abiotic surfaces. Importantly, biofilms provide protection from antibiotics and the host immune system. Bacterial biofilms on implant material is considered as the leading cause of the tissue destruction resulting in osteolysis and implant loosening. Infection persists for longer duration because biofilms escape the host defence mechanisms. Biofilm formation in *S. aureus* is seen due to a polysaccharide intercellular adhesion (PIA) and because of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)· [8-11] *S. aureus* initial attachment to both host tissues and biomaterials are due to these materials.[12] MSCRAMMs play a vital role in initiation of endovascular, bone and joint and prosthetic device infections.[13] Biofilms by rendering antibiotics ineffective by not allowing them to penetrate and making the strain insensitive for host immune system leading the bacteria towards higher virulence.

The aim of this study was to compare the prevalence of biofilm formation and its effect on virulence MRSA isolates by enhancing resistance for antibiotics.

METHODS

Bacterial isolates

A total of 221 *S. aureus* clinical isolates were collected from various clinical specimens including urine, pus, blood, sputum, stool, semen, throat swab and different body fluids, received routinely in microbiology lab of HAHC hospital, Jamia Hamdard University, New Delhi over a period of one year from January 2017 to December 2017. The study was approved by ethical committee of HIMSR, Jamia Hamdard. (Table 1)

Table 1: Quantitative distribution of *staphylococcus aureus* in different clinical samples.

Sample	Number (n=221)	Percentage (%)
Pus	149	67.42
Blood	29	13.12
Urine	29	13.12
Ear swab	5	2.26
Sputum	3	1.35
Ascitic fluid	2	0.94
Tissue	2	0.90
Cyst	1	0.45
Semen	1	0.45
Total	**221**	

Study type

Study type was cross sectional study.

Inclusion criteria

All samples from which *S. aureus* were isolated.

Exclusion criteria

Samples not collected with aseptic techniques and falling under rejection criteria of lab.

Sample size

Sample Size was calculated with the help of confidence level, confidence interval and targeted population by using survey software. Where confidence level was 95%, confidence interval was 5% and the targeted population was 60000. As study was conducted at a tertiary care hospital having patients from highly crowded area between 10 kms.

Identification

The isolates were identified using biochemical tests, such as mannitol fermentation, slide and tube coagulase tests, colony morphology on blood agar medium and VITEK 2 automated identification system.[14]

Detection of MRSA

MRSA strains were detected by using 30µg cefoxitin disc (HI media Mumbai), and confirmed by measuring size of zone of inhibition as per CLSI 2016. [15] (table 2)

Table 2: Detection of MRSA.

Antibiotics	Resistant	Sensitive
Cefoxitin(30µg)	≤21mm	≥22mm

Antibiotic susceptibility test

Antimicrobial susceptibility test (AST) was performed by Kirby Bauer disc diffusion method and by using VITEK 2 automated sensitivity testing machine. The *S. aureus* strain of ATCC 25923 was used as a control the quality of the antibiotic susceptibility testing. Different antibiotics of different class were used in AST, including benzylepenicillin, oxacillin (1 µg), erythromycin (15 µg), clindamycin (2 µg), vancomycin (2 µg), linezolid (30 µg), trimethoprim-sulfamethoxazole (25 µg), gentamicin (10 µg), and ciprofloxacin (5 µg). [15]

Microtiter plate (MTP) method for detection of biofilm production

MRSA isolates were grown overnight at 37ºC in brain heart infusion broth. This culture was diluted 1:100 in medium. 150 µl was used to inoculate sterile flat-bottomed 96 well micro titer plates. Incubate for 48 hours at 37ºC . Wells were gently washed three times with distilled water. Plate was then dried in an inverted position, and stained with 300µl of 2% crystal violet solution in water for 45 min. Again plate was washed three times. Quantitative analysis of biofilm production was performed by adding 200µl of ethanol-acetic acid (95:5, vol/vol) to destain the wells. From each well 100 microliters was transferred to a new micro titer plate, and using a micro titer plate reader the level; (OD) of crystal violet was measured , in the destaining solution at 570nm. Each assay was performed in triplicate. Uninoculated medium was used as a control. The mean OD_{570} value from the control wells was subtracted from the mean OD_{570} value of tested well. [16]

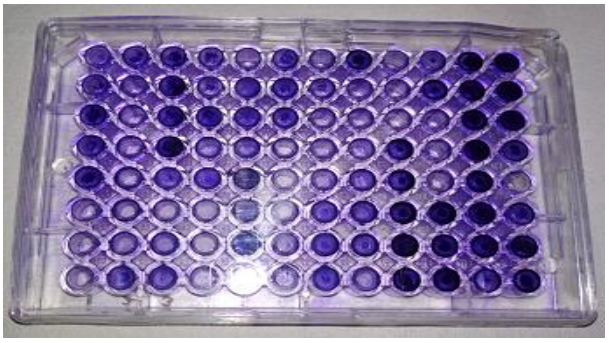

Figure 1: Biofilm Production Criteria.

Table 3: Analysis of Biofilm formation.

O.D.	Biofilm
<0.20	Negative
0.20-0.50	Positive (Low Biofilm Formation)
>0.50	Positive (High Biofilm Formation)

Data analysis

Pearson's chi-square was used to in statistical analysis. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Prevalence of MRSA and MSSA

Out of 221 strains of *Staphylococcus aureus*, 113 (51.13%) strains of MRSA and 108 (48.86%) strains of MSSA were isolated. (Table 4)

Table 4: Prevalence of MRSA and MSSA in clinical isolates.

Strains	No. of samples (N=221)	Percentage (n/Nx100)
MRSA (n)	113	51.13
MSSA (n)	108	48.86
Total strains	221	100

Antibiotic susceptibility testing

All the isolates either they are MRSA or MSSA tested for all ten antibiotics and we found a remarkable resistance difference between both. (Table 5)

Table 5: Antibiotic resistance of MRSA and MSSA strains.

Antibiotics	MRSA	MSSA
Benzylepenicillin	113(100%)	104(96%)
Oxacillin	113(100%)	0(0%)
Ciprofloxacin	103(91.10%)	89(82.40%)
Erythromycin	89(78.76%)	43(39.81%)
Trimethoprim/ sulfamethexazole	83(73.45%)	65(60.18%)
Clindamycin	48(42.47%)	18(16.50%)
Gentamycin	33(29.20%)	11(10.18%)
Linezolid	9(7.90%)	0(0%)
Tigecycline	7(6.19%)	0(0%)
Vancomycin	0(0%)	0(0%)

Comparison of Antibiotic resistance in biofilm producer and non-biofilm producer.

It was observed that antibiotic resistance was higher in biofilm producing strains of MRSA and MSSA than non-biofilm producers respectively. (Table 6)

Table 6: Comparison of antibiotic resistance among Biofilm producing and non-biofilm producing strains.

Antibiotics	High Biofilm Producer (n=38)%	Low Biofilm Producer (n=14)%	No Biofilm Produces (n=61)%
Benzylepenicillin	38(100)	14(100)	61(100)
Oxacillin	38(100)	14(100)	61(100)
Gentamycin	10(26.31)	4(28.57)	19(31.14)
Ciprofloxacin	37(97.3)	13(92.8)	53(86.88)
Erythromycin	35(92.10)	11(78.57)	43(70.49)
Clindamycin	21(55.26)	8(57.14)	19(31.14)
Linazolid	5(13.15)	1(7.14)	3(4.91)
Vancomycin	0(0)	0(0)	0(0)
Tigecycline	4(10.5)	1(7.14)	2(3.2)
Trimethoprim/ sulfamethexazole	24(63.15)	9(64.28)	50(81.96)

DISCUSSION

S. aureus has been associated with many community as well as nosocomial infection. Now a days hospital infection due to MRSA is an emerging problem and it is difficult to treat these infections. Beyond 1961 *Staphylococcus aureus* has shown a linear increase in resistance development. Surveys are important in determining optimum empirical therapy for severe infections. *S.* aureus possesses many virulence factors that enable the organism to take advantage of a compromised host but biofilm producers make them virulent for even healthy individuals as host immune response becomes ineffective.[3,4,12]

The overall prevalence of MRSA strains in our study was found to be 51.13%. Similar results of 40-50% were reported in other studies .[17,18] Although lower prevalence has been reported (42%) and (40%) among different isolates in 2008 and 2009 respectively.[19]K. Rajaduraipandi has also reported lower prevalence rate of 31.1% from Tamil Nadu, India.

Healthcare system has shown remarkable progress by innovating implantable medical devices. Biofilm-associated infections with *S. aureus* are the most common cause of device related infections. To enhance the problem, biofilms infections are particularly difficult to treat as bacteria within the matrix are more resistant to antimicrobial agents and the host immune response. MRSA biofilms showed vital role in many chronic infections. The gene *spa* type t127 is found to be associated with biofilm formation in community-acquired MRSA. Moreover, a number of strains possess a many resistance mechanisms against conventional antibiotics.

In our study 113 isolates of MRSA were tested for biofilm production, 52(46.02%) were identified as biofilm producers. Low biofilm production was found in 14(12.38%) isolates and 38(33.63%) were found as high biofilm producers. In other studies conducted 64.9%, 77% and 95.4 % biofilm production rate has been reported. In comparison to MRSA, MSSA isolates have lesser ability to produce biofilm.[19]As it was time bound study sample size was not very large so our results may be showing little variation. Also due to financial constraint molecular test could not be performed otherwise genes for biofilms and resistance could be correlated.

It has been reported that biofilms increase resistance to external agents, antibiotics, and internal agents as our immune system. The first is prevention from reaching their target.[20-21] Secondly, by changing the physiology of biofilm-dwelling bacteria. Cells within the biofilm are in slow-growers. The slow growth rate of persister cells affects the efficacy of antibiotics, which target active cell processes.[22-24] In our study we found that biofilm producers are more resistant to antibiotics like reported by other studies.[25]

CONCLUSION

Rising MRSA infection rates pose a significant risk to human health. While increasing antibiotic resistance is a well appreciated contributing factor, a lesser appreciated but more important factor is the ability of S. aureus to form biofilms. As biofilm-dwelling bacteria are generally able to tolerate much higher antibiotic concentrations biofilm-associated infections are difficult to eradicate.

Most chronic MRSA infections reveal the biofilm state in their pathogenesis. This is especially true for those associated with indwelling medical devices. As most therapeutic strategies are only effective at treating planktonic cells or acute infections, there is an urgent need to develop new therapeutic strategies capable of targeting S. aureus in the biofilm state.

While many new approaches to eradicate S. aureus biofilms have been tried over the past two decades such as small molecules that prevent biofilm formation, enzymes that weaken biofilm matrix structural integrity,

and antibodies and vaccines that target specific biofilm life cycle stages, but these approaches have to be clinically validated.[18]

Research like identifying cationic small molecules with exclusive antibiofilm activity, human milk oligosaccharides (HMOs), non-conjugated oligosaccharides abundant in human milk, found to modulate growth and biofilm production for several bacterial pathogens, including MRSA[26] should be done at higher level. Further how bacteria coordinate the expression of various effectors and how surfaces react with these effectors will be required to know to the development of antibiofilm compounds. Knowledge of all this has the potential to identify bacterial targets that can be engaged to target biofilm production selectively without accompanying antimicrobial activity.

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