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Original Article

Biofilm formation and antibiotic resistance in Methicillin Resistant Staphylococcus aureus among nasal isolated of HIV infected patients in a tertiary care hospital

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ABSTRACT:

Background: Biofilm production is an important virulence factor of S. aureus. HIV is an established risk factor for MRSA nasal carriage and associated infections. MRSA isolates are more prone to form biofilm which may be a therapeutic emergency in HIV positive patients. **Methods:** HIV positive patients were taken as cases and Negative as Controls group. Antibiotic resistance was done by Kirby Bauer disc diffusion method. Biofilm formation was detected by Tissue culture plate method, Tube method and Congo red agar method. **Result:** Out of 96 S. aureus isolates 18 (18.75%) isolates were found to be Methicillin resistant in which 14 (78%) were biofilm producers while 4 (22%) isolates of MRSA were non biofilm producers by TCP method. Among HIV positive case group; 22% & 56% MRSA isolates were strong & moderate biofilm producer while, none of the MRSA isolate was strong biofilm producer among HIV Negative control group isolates. Tissue culture plate method was found 100% specific & 100% sensitive and 100% accurate method as compared to other methods. Chi square test for antibiotic resistance of Biofilm producer MRSA among HIV positive and HIV Negative groups showed significant difference for all the 16 antibiotics enlisted. All the isolates showed 100 % sensitive to Vancomycin, Teicoplanin and Linezolid while resistance against Amikacin, gentamycin and Tetracycline was shown only by biofilm producers. **Conclusion:** We recommend the TCP method as "Gold Standard" for biofilm detection in routine screening procedures.

Keywords: Biofilm, Methicillin resistance, HIV, Nasal carriage, Antibiotic resistance.

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INTRODUCTION

Staphylococci are recognized as the most frequent causes of biofilm-associated drug resistant infections in community and hospitals all over the world. Biofilm production is well known virulence factor of *S. aureus*.[1, 2] The ability of biofilm formation and possibility of extensive epidemic with multiple drug resistant MRSA might be difficult to control.[3] MRSA carriage is more frequent in HIV- positive individuals than HIV non-infected individuals. Positive nasal carriage of Staphylococcus specially MRSA may have a role on further opportunistic infections in HIV positive patients; which is a potential risk factors for community acquired MRSA.[4-7] Nasal carriage provides a staging ground to disseminate S aureus to other sites of the body and comes into the circulation through an epithelial breach or planktonic growth. Host innate immune response either removes the organism or it attaches to the host extracellular matrix proteins and form a biofilm.[1] The formation of biofilm is result of a phenotypic change in *S. aureus* to adapt to its surroundings in the presence of environmental challenge.[8]

A biofilm can be defined as a 'microbially-derived sessile community, typified by cells that are attached to a

substratum, interface, or to each other, are embedded in a matrix of extracellular polymeric substance, and exhibit an altered phenotype with regard to growth, gene expression and protein production'.[9]

Various authors demonstrated that biofilm producing isolates had a higher incidence of multi-resistance than biofilm non-producers from the same population.[10-13]

Biofilm formation is a bacterial survival strategy through which S.aureus can persist in clinical settings and gain increased resistance to antimicrobial agents. [9, 14] Therefore, biofilm producing MRSA becomes multi drug resistant and difficult to treat.[15] It has been observed that about 65% of the nosocomial infections are associated with biofilm formation[16-17] and 10 to 1000 times more difficult to treat with an empirical treatment.[18]

In a study in Lucknow, a significant association between antibiotic resistance and biofilm production in staphylococci was reported.[8, 19] Biofilm makes S.aureus mare persistent and boosts its levels of antimicrobial resistance, also against natural AMPs present in the host's nasal mucosa.[20] Hence host innate immunity is effectively compromised by immune-evasion strategies of the nasal carrier strain. [21] Reports by various authors from different geographical areas show a great diversity in the prevalence of nasal carriage MRSA among the HIV seropositive patients ranging from 2-53%. [6, 22]

The identification of possible associations between biofilm formation and antibiotic resistance of nasal isolates of *MRSA* could provide better control measures particularly among HIV infected individuals. Therefore, we have planned this study to determine the prevalence of nasal carriage of MRSA in HIV seropositive patients and to find out the role of biofilm formation on antibiotic resistance

MATERIALS AND METHODS

In this study, nasal swab samples from 220 newly diagnosed HIV seropositive patients attending ICTC were taken as cases and healthy persons were included as control after obtaining written consent. All nasal swab specimens were cultured aerobically on blood agar & Mannitol salt agar (Hi Media, India) for 24 hrs at 37^0C. S aureus were identified and differentiated from related organisms as per conventional methods on the basis of colony morphology, Gram staining, catalase, coagulase, DNAse and mannitol fermentation test following the standard procedures.[23]

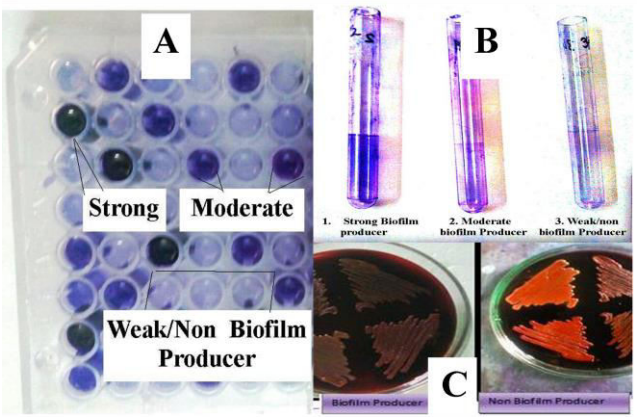
Antibiotic sensitivity testing:

The antibiotic susceptibility pattern for selected antibiotics was performed by Kirby Bauer disc diffusion method and interpretated according to CLSI guidelines. [24] All the staphylococcus aureus isolates were tested for Methicillin resistance by Cefoxitin disc diffusion method, Oxacillin screen agar (OSA) and finally confirmed by mecA gene detection by PCR.

Detection of Biofilm Formation:

Biofilm formation was detected by Tissue culture plate method (TCP), Tube method and Congo red agar (CRA) method. Staphylococcus aureus ATCC 25923 was taken as Biofilm Positive control and Staphylococcus epidermidis ATCC 12228 was taken as Biofilm Negative control. Grading of biofilm formation is shown in Figure-1.

Figure 1: Grading of biofilm formation by different methods



A- Grading by Tissue culture Plate (TCP) method, B- Tube method, C- Congo red Agar method

Tissue culture plate method (TCP):

Biofilm formation by TCP method was done as described earlier by Kwon et al.[25] and T Mathur et al.[26] by using polystyrene microtitre plates (Tissue culture Plate) with brain heart infusion (BHI) broth supplemented with 1% sucrose. Prior to inoculate the plate, the microorganisms were grown in BHI for 2 to 4 hours. 199 µl of the 1% sucrose - brain heart infusion was placed to all wells of the microtitre plate and 1 µl of the microbial suspension was added in designated wells in duplicate. The plate was sealed and incubated at 37°C for 24 hours, then five times washed with phosphate buffered saline (PBS) to remove any unfixed microbial cell. Then 175 µl of 1% crystal violet was added to all wells and incubated at room temperature for 15 min. The plates was further washed 5 times with PBS and dried for 30 min at room temperature. Then the wells were destained by 200 µl of ethanol-acetone (80:20 v/v) and incubated at room temp for 25 min. Now, 100 µl of destained solution from each well were placed in a new sterile flat-bottomed 96-well poly styrene micro-titer plates in their respective position and absorbance was calculated in an Elisa reader at 570 nm. These OD values were considered for an index of bacteria adhering to surface and biofilm formation. OD<O.120 were considered for weak biofilm producers. Optical Densities (OD) values between O.120 - 0.240 were considered for moderate biofilm producers and OD>O.240 was considered strong biofilm producers. In Figure- 1(A), the intensity of colored well is showing the grading of biofilm formation.

TUBE METHOD (TM):

Biofilm formation was determined by Tube method as described by Christensen et al (1985) with some modifications.[27] Brain Heart Infusion supplemented with

1% sucrose was inoculated with freshly cultured nasal isolates and incubated aerobically at 37°C for 24 hours in a sterile test tube. Then the tubes were washed with PBS (pH 7.3) gently. Further washed tubes were stained with 0.1% crystal violet for 15 minutes then washed with distilled water. Tubes were then air-dried by keeping in inverted position. A dark purple colored layer formed on the wall and bottom of the tube were considered as biofilm positive. Circle layer formation at the liquid interface inside the tube was not indicative of biofilm formation at all. Biofilm formation was scored as absent/weak, moderate and strong on the basis of the intensity of the crystal violet adhered on the wall of the tube. In Figure- 1(B), the intensity of colored tube is showing the grading of biofilm formation.

CONGO RED AGAR (CRA) METHOD:

Biofilm detection by Congo Red Agar was performed as per the method described by Mathur et al.[26] by using Congo red agar media; composed of BHI broth (37 gms/L), Agar powder (10 gms/L), sucrose (50 gms/L) and Congo red dye (0.8 gms/L). The CRA Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C.

Growth of black colonies with a dry crystalline appearance indicates Positive result for biofilm production whereas; pink/red colonies were observed as Weak biofilm producers. The dark colonies without dry crystalline colonial morphology indicated an indeterminate result. In Figure- 1(C), biofilm formation is shown by the dark crystalline color developed on the agar plate.

STATISTICAL ANALYSIS:

Statistical Package for the Social Sciences (SPSS) software (IBM SPSS Data Access pack 7.1) was used for data analysis. Chi-square test was used for analysis of data & P-value < 0.05 was set as statistical significance level.

RESULT:

The biofilm formation was carried out by TCP method, Tube method and CRA Method; among which TCP method was found 100% specific (95% CI- 95.89% to 100.00%) , 100% sensitive (95% CI- 97.24% to 100.00%) and 100% accurate (95%CI- 98.34% to 100.00%) method as compared to other methods. Congo Red Agar method showed good specificity (99.24%; 95.85% to 99.98%, 95%CI) but poor sensitivity (31.82%; 22.29% to 42.61%, 95% CI). Tube method showed average sensitivity (79.55%; 69.61% to 87.40%, 95% CI) & good specificity (98.48%; 94.63% to 99.82%, 95% CI). Nasal isolates among HIV positive patients showed 40% s. aureus & 78% MRSA were biofilm produce, where as 10% & 33% respectively in HIV negative control group. Among HIV positive case group; 22% MRSA isolates were strong biofilm producer while, none among control group isolates. The observation for detection of Biofilm formation in Nasal isolates of MRSA by different methods among HIV positive cases & HIV negative Controls is shown in Table-1.

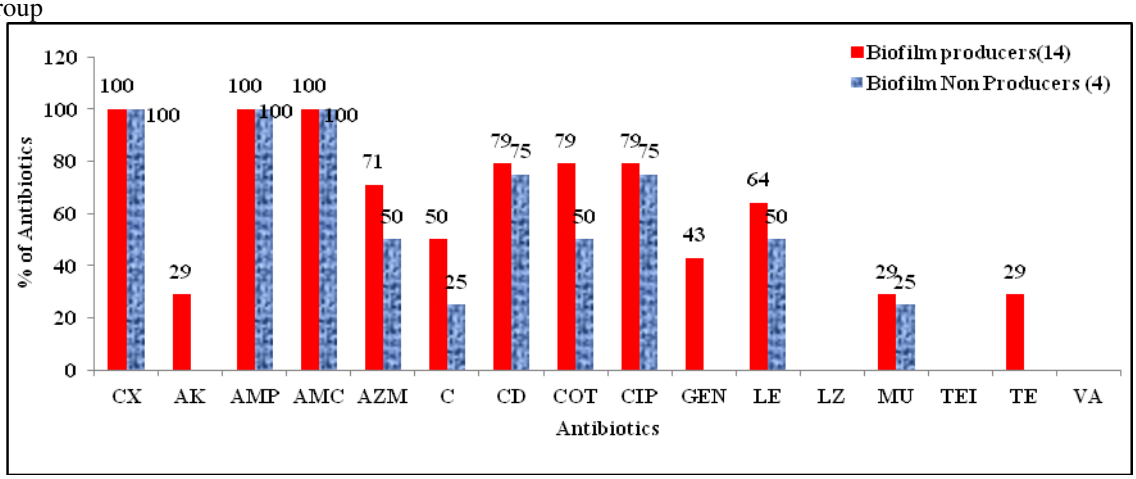
Table-1: Detection of Biofilm formation in Nasal isolates of MRSA by different methods among HIV positive cases & HIV negative Controls

	TCP Method			Tube Method			CRA Method	
Bio Film Production	Strong (%)	Moderate (%)	Weak / Non (%)	Strong (%)	Moderate (%)	Weak / Non (%)	Producer (%)	Non Producer (%)
MRSA-18 (HIV +)	4(22)	10(56)	4(22)	3(17)	9(50)	6(33)	5(28)	13(72)
MSSA-78 (HIV +)	8(10)	16(21)	54(69)	5(6)	19(24)	54(69)	12(15)	66(85)
(Control) MRSA (03)	0	1(33)	2(67)	0	0	3(100)	0	3(100)
(Control) MSSA (45)	0	4(9)	41(91)	0	4(9)	41(91)	0	41(91)

Antibiotic –resistance profile of MRSA: Among HIV positive patients; out of 96 S. aureus isolates 18 (18.75%) isolates were found to be Methicillin resistant in which 14 (78%) were biofilm producers while 4 (22%) isolates of MRSA were non biofilm producers. Biofilm producers and Non producers exhibited diverse resistant profile against 16 commonly used antibiotics (Figure 2).

All the isolates showed 100 % sensitive to Vancomycin, Teicoplanin and Linezolid while resistance against Amikacin, gentamycin and Tetracycline was shown only by biofilm producers. Chi square test showed statistically significant resistance between biofilm producers & Non Producer among nasal isolates of MRSA in HIV positive case group for Amikacin (P= 0.0021), Azithromycin (P= 0.0026), Chloramphenicol (P=0.0003), Cotrimoxazole (P= < 0.0001), Levofloxacin (P=0.0463), Gentamycin (P=0.0005), Tetracycline (P=0.0021) while no statistically significant difference was observed against Clindamycin (P= 0.50), ciprofloxacin (P= 0.50) and mupirocin (P= 0.52). Chi square test for antibiotic resistance of Biofilm producer MRSA among HIV positive and HIV Negative groups showed significant difference for all the 16 antibiotics enlisted.

Figure 2: Antibiotic resistance of Nasal carriage MRSA in Biofilm producers & Non Producer among HIV positive case group



(CX- Cefoxitin, AK- Amikacin, AMP- Ampicillin, AMC- Amoxicillin clavulanic acid, AZM-Azithromycin, C- Chlomphenicol, CD- Clindamycin, COT- Cotrimoxazole, CIP- Ciprofloxacin, GEN- Gentamycin, LE-Levofloxacin, LZ- Linezolid, MU- Mupirocin, TEI- Teicoplanin, TE- Tetracycline, VA- Vancomycin)

DISCUSSION

MRSA is a serious and dreadful challenge as their prevalence is reported to be increasing exponentially.[28] *Staphylococcus aureus* is well established opportunistic pathogen among HIV and AIDS patients and a frequent cause of morbidity.[22,29-30] However, very few studies have been carried out on nasal isolates of MRSA among HIV positive patients especially, specially biofilm formation among staphylococcal nasal isolates.

Biofilm formation has been described as a potential virulence factor of *S. aureus*.[1, 31] MRSA have the ability to form biofilm and tend to be Multidrug resistance; causes various severe systemic and opportunistic infections. So, the screening for MRSA nasal carriage in immunocompromised patients with the ability of biofilm forming might help in therapeutic scarcity in the community.[32-34]

In our study, 40% of S.aureus isolates and 78% of MRSA isolates among HIV positive patients had the ability to form biofilm as compared to control group (10% & 33%) by TCP method which was similar to observations reported by various researchers [35-37] and contradictory to the observation made by Solmaz Ohadian Moghadam et al.[38] and Samie, A. and Shivambu.[39]

We found 22% MRSA as strong biofilm producer and 56% moderate biofilm producer which is slightly similar to findings of Maryam Rezaei et al.[40] and Khairalla AS et al.[41] while, contradictory findings was reported by Dardi Charan Kaur et al. [37]

In tube method, 67% MRSA isolates were found to be biofilm producers which varied from other studies. [42] Similarly, Nahla A. Melake et al.[35] found much more (76.6%) slime layer producer MRSA than our findings (28%) by CRA method. We used three different methods to detect biofilm formation. Tissue culture plate method was found 100% specific, sensitive and accurate method as compared to TM and CRA methods. So, it is considered to be best in detecting biofilm formation. The tube method correlates precisely with the TCP method for strong biofilm producers but, it was difficult to discriminate between weak and negative isolates. CRA method did not correlate well with other methods. Tissue Culture Plate method was found to be most sensitive, accurate and reproducible screening method for detection of biofilm formation which is also advocated by various authors and may be used as Gold standard in routine.[26, 38, 40, 43-44]

However, majority of the authors reported TCP Method as gold standard to detect the biofilm formation in routine; some researchers suggests Tube Method as a better tool [45-46] for this while, some suggests CRA method as better tool[47] and may be used as an alternative phenotypic test of TCP method.[8, 18]

Moreover, in the present study, 78% of MRSA nasal isolates were biofilm producers in this region among newly diagnosed HIV positive patients. These isolates exhibited diverse resistant profile against 16 commonly used antibiotics (Figure 2). This observation was also supported by other studies.[9, 42] All the isolates showed 100 % sensitive to Vancomycin, Teicoplanin and Linezolid while resistance against Amikacin, gentamycin and Tetracycline was shown only by biofilm producers. Antibiogram reported by Maryam Rezaei et al.[40] for biofilm producer MRSA isolates was almost similar to our findings except erythromycin (64% vs. 100%), tetracycline & Amikacin (0% vs. 29%). Murugan S et al.[45] also found 100% sensitive to Vancomycin and significant difference in antibiogram between biofilm producer & Non Producer isolates like our observation. Whereas, Nahla A. Melake et al.[35] reported an increased resistance among biofilm producer Nasal MRSA isolates against Vancomycin & Teicoplanin.

Chi square test showed statistically significant resistance between biofilm producers & Non Producer among nasal isolates of MRSA in HIV positive case group for Amikacin, Azithromycin, Chloramphenicol, Cotrimoxazole, Levofloxacin, Gentamycin, Tetracycline; while no statistically significant difference was observed against Clindamycin, ciprofloxacin and mupirocin. Antibiotic resistance of Biofilm producer MRSA among

HIV positive and HIV Negative groups showed significant difference for all the 16 antibiotics enlisted. However, Samie A. and Shivambu[39] didn't found any significant difference for resistance against biofilm producer and Non producer S.aureus isolates among HIV patients. This result was contrast to our findings, however near to similar finding was also reported.[42, 48]

Since biofilm forming ability increase the resistance to commonly used antibiotics, and HIV is a risk factor for Nasal carriage and S. aureus infections; screening of biofilm forming MRSA nasal carrier that can be easily transmitted to/from other people in the community and hospital; is necessary for public health and would definitely provide better control measures. Judicious use of antibiotics and regular screening of nasal carriage MRSA and biofilm producing ability by them will definitely provide a guideline to treat and prevent the staphylococcal infection in HIV patients.

CONCLUSION

We strongly propose the regular monitoring of nasal carriage of S.aureus in the HIV seropositive patients and biofilm formation by TCP method along with other routine investigations.

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