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EVALUATION OF DIFFERENT DETECTION METHODS OF BIOFILM FORMATION IN CLINICAL ISOLATES OF STAPHYLOCOCCI

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ABSTRACT

In this study 400 clinical isolates of staphylococci which include 250 isolates of S. aureus and 150 isolates of Coagulase negative staphylococci (CoNS) were screened for biofilm production. They were tested by three methods, Tissue culture plate (TCP) method, Tube method and Congo red agar (CRA) method. TCP method detected 105 (26.3%) isolates as strong biofilm producers, 68 (17%) as moderate biofilm producers and 227 (56.8%) as non producers of biofilm. Tube method and CRA method could detect 20.5% & 25.3% as strong producers and 63.8% and 74.8% as non producers respectively. TCP method could detect biofilm production in 45.6% of *S.aureus* and38.7% of CoNS. Maximum biofilm producing staphylococci was isolated from patients with artificial devices and the most common isolate was *S.epidermidis*. Biofilm production was more in Methicillin resistant strains and they showed higher degree of resistance to almost all the groups of antibiotics.

KEY WORDS: Staphylococci, MRSA, Biofilm detection, Tissue culture plate method, Congo red Agar.

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INTRODUCTION

Staphylococci are recognised as important cause of disease around the world. Staphylococcal infections are of particular concern because of the causative agent offering resistance to a wide range of antibiotics. Infections due to multiple drug resistant strains are becoming more critical due to their capacity to produce biofilm. This slime or biofilm consists of layers of cell clusters embedded in a matrix of extra-cellular polysaccharide called Polysaccharide Intercellular Adhesin (PIA). PIA is involved in cell to cell adhesion and is essential for biofilm production.

REVIEW OF LITERATURE

Biofilm helps the bacteria to form stable communities of protection rather than live as free planktonic cells[1]. It also impedes delivery of antibiotics[2]. Biofilms can resist antibiotic concentration 10 – 10,000 folds higher than those required to inhibit the growth of free floating bacteria[3]. These infections are generally associated with the use of catheters and other medical devices. Infectious processes in which biofilms are implicated include common problems such as UTI, catheter infections, middle ear infections, dental plaque, coating contact lenses and less common but more lethal processes such as infective endocarditis, infections of joint prosthesis and heart valves[4,5]. Biofilm producing staphylococci have also been isolated from various clinical samples like blood, urine, pus, skin surface etc[6,7]. The differentiation of staphylococci with respect to its biofilm phenotype might help to elucidate the impact of staphylococci in diagnosis and these observations may have utility in the prevention of infections[8].

Various methods are currently used in medical areas for the detection of biofilm production. These methods include visual assessment by electron microscopy and molecular techniques such as polymerase chain reaction. However, qualitative methods such as tube adherence test described by Christensen's et al. Congo Red Agar (CRA) method described by Freeman et al. and quantitative method such as the Tissue Culture Plate (TCP) method described by Christensen et al. are used in routine laboratories[9,10,11].

AIMS AND OBJECTIVES

The present study was undertaken to detect prevalence of biofilm production in staphylococci in a tertiary care hospital in Navi Mumbai and to evaluate three different methods i.e. Tube Method (TM), Congo Red Agar Method (CRA) and Tissue Culture Plate Method (TCP) for their detection and to assess the relation of biofilm formation with methicillin resistance and anti-microbial resistance.

MATERIALS AND METHODS

After obtaining ethical clearance, a total of 400 non-repetitive clinical strains of staphylococci were isolated from various clinical samples of indoor and outdoor patients.

All the isolates were identified using standard microbiological procedures as per the guidelines of Kloos and Schleiffer's scheme and Coneman's dichotomous key [12,13]. They were subjected to antibiotic susceptibility test by Kirby Bauer's disc diffusion method on Mueller Hinton (MH) agar and the zones were interpreted as per CLSI guidelines[14,15]. Antibiotic discs with their respective potencies were as under

Antibiotic	Symbol	Potency
Penicillin G	P	10 units
Cloxacillin	CX	1mcg
Ampicillin+Sulbactam	AS	10/10 mcg
Amoxicillin+Clavulanic acid	AMC	20/10 mcg
Erythromycin	E	15mcg
Roxithromycin	AT/RF	15mcg
Ciprofloxacin	CF	15mcg
Leavofloxacin	QB	5mcg
Cephalexin	PR	30mcg
Cefotaxime	CE	30mcg
Gentamicin	G	10mcg
Lincomycin	LM	2mcg
Co-trimoxazole	BA	1.25+23.75mcg
Tetracycline	TE	30mcg
Vancomycin	VA	30mcg
Teicoplanin	TE	30mcg
Linezolid	LZ	30mcg

Methicillin resistance was checked using oxacillin discs(1mcg) on 4% salt agar as per CLSI guidelines and cefoxitin discs (30mcg)on MH agar and the strains were identified as Methicillin resistant *S. aureus* (MRSA), Methicillin sensitive *S. aureus* (MSSA), Methicillin resistant Coagulase Negative Staphylococci (MRCoNS) and Methicillin sensitive Coagulase Negative Staphylococci (MSCoNS).

Detection of Biofilm

1) Tube Method (TM):- Biofilm production was investigated by the tube adherence test proposed by Christensen et al[9]. Ten ml Trypticase soya broth with 1% glucose was inoculated with the test organism on nutrient agar individually. Broths were incubated at 37 ºC for 24 hours. The cultures were aspirated and the tubes were washed with phosphate buffer saline pH 7.3. The tubes were dried and stained with safranine or 0.1% crystal violet. Excess stain was removed. Tubes were dried in inverted position. In positive biofilm formation, a visible stained film was seen along the walls and bottom of the tube.(Figure-1)

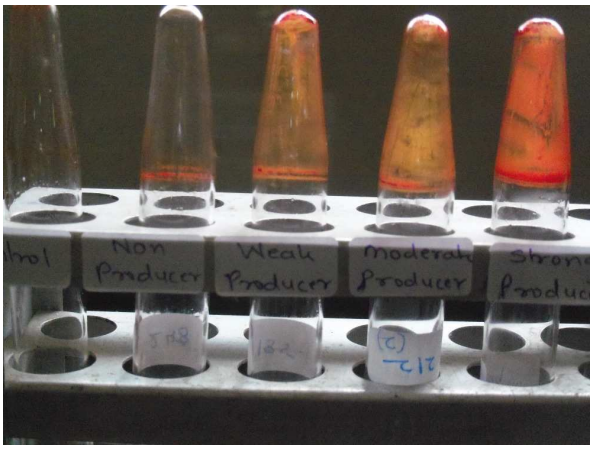

Figure-1
Biofilm production by Tube Method.

2) Congo red agar (CRA) method (Freeman et al.[10]) :- Congo red agar (CRA) medium was prepared with brain heart infusion broth, sucrose, agar and Congo red indicator. Congo red stain was prepared separately in sterile distilled water and was added to sterile molten agar base and then the medium was poured in the plates. CRA plates were inoculated with the test organisms and incubated at 37 ºC for 24 hours to 48 hours aerobically. Black colonies with dry crystalline consistency indicate strong biofilm formation. Brownish or reddish growth was considered as negative biofilm formation. (Figure-2).

Figure-2
Biofilm production on Congo Red Agar

3) Tissue Culture Plate (TCP) Method (Eftekhar and Speert [16]):- Overnight growth of bacteria in Trypticase soya broth (TSB) with 1% glucose was diluted 1 : 100 and 200 µl portions were inoculated in 96 - well flat bottom polystyrene microtitre plates (Nuclon TM Ltd). Incubation was carried out at 35 ºC for 24 hours. Cultures were then aspirated and the wells were washed 3 times with phosphate buffer saline pH 7.2. The plates were then air-dried overnight and stained with 0.1% safranine. The optical density of the wells was measured at 490 nm using micro ELISA auto reader. Robonic- Readwell touch –Automatic ELISA plate analyser).An optical density of 0.12 was chosen to distinguish biofilm producers from those that did not form biofilm. Sterile TSB was used as a negative control (Blank). To compensate for background absorbance, the OD reading value of blank was deducted from the test values. Intensity of Biofilm was classified as given by Mathur et al.[17] (Figure-3).

OD Value	Biofilm Formation
< 0.120	Non Biofilm producer
0.120 – 0.240	Moderate Biofilm producer
> 0.240	Strong Biofilm producer

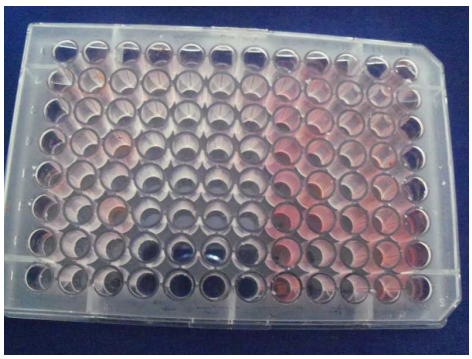

Figure 3
Biofilm production by TCP Method.

RESULTS

Table 1
Detection of Biofilm in Staphylococci by three phenotypic methods

	S. aureus (250)		CoNS (150)		Staphylococci (400)	
	MRSA (73)	MSSA (177)	MRCoNS (50)	MSCoNS (100)	Total (400)	% Positive
Tube method (Strong)	36	21	16	9	82	20.5%
%	43.9%	25.6%	19.5%	11.0%		
Tube method (moderate)	18	27	8	10	63	15.8%
%	28.6%	42.9%	12.7%	15.9%		
Tube method (Negative)	19	129	26	81	255	63.8%
%	7.5%	50.6%	10.2%	31.8%		
Total Positive	54	48	24	19	145	36.3%
%	37.2%	33.1%	16.6%	13.1%		
CRA (Strong producers)	40	28	21	12	101	25.3%
%	39.6%	27.7%	20.8%	11.9%		
CRA (Non producers)	33	149	29	88	299	74.8%
%	11.0%	49.8%	9.7%	29.4%		
Total Positive	40	28	21	12	101	25.3%
%	39.6%	27.7%	20.8%	11.9%		
TCP (Strong) OD > 0.240	42	30	23	10	105	26.3%
%	40.0%	28.6%	21.9%	9.5%		
TCP (Moderate) OD = 0.12-0.24	17	26	7	18	68	17.0%
%	25.0%	38.2%	10.3%	26.5%		
TCP (Negative) OD <0.12	14	121	20	72	227	56.8%
	6.2%	53.3%	8.8%	31.7%		
Total Positive	59	56	30	28	173	43.3%
%	34.1%	32.4%	17.3%	16.2%		

% values indicate percentage of the biofilm positive isolates in the total number of isolates.

Table 2
Biofilm production in Staphylococci isolated from various specimens (TCP method)

Specimens	Total Specimens	Biofilm non-producers	Biofilm Producers	% (Biofilm producers)
Pus	220	126	94	42.7%
Blood	74	40	34	45.9%
Urine	44	31	13	29.5%
Sputum	19	14	5	26.3%
Miscellanious	24	15	9	37.5%
Artificial Divices	19	2	17	89.5%
Total	400	228	172	43.0%

Table 3
Biofilm production by TCP method in various isolates of staphylococci

Organism	Total Isolates	Biofilm Producers by TCP	%
S.aureus	250	114	45.6%
CoNS	150	58	38.7%
S.epidermidis	82	44	53.7%
S.haemolyticus	16	8	50.0%
S.lugdunensis	17	3	17.6%
S.Schleifferi	14	3	21.4%
S.saprophyticus	5	0	0.0%
S.warneri	10	0	0.0%
S.capitis	1	0	0.0%
S.hominis	3	0	0.0%
S.xylosus	2	0	0.0%

% values indicate percentage of the biofilm positive isolates in the total number of isolates.

Table 4
Detection of Biofilm (TCP method) in Methicillin Resistant and Sensitive isolates of Staphylococci

	S. aureus (250)		CoNS (150)		Staphylococci
	MRSA (73)	MSSA (177)	MRCoNS (50)	MSCoNS (100)	Total (400)
Total MRSA	73	177	50	100	400
Total Biofilm producers	59	56	30	28	173
%	80.8%	31.6%	60.0%	28.0%	43.3%
Total Biofilm non - producers	14	121	20	72	227
%	19.2%	68.4%	40.0%	72.0%	56.8%

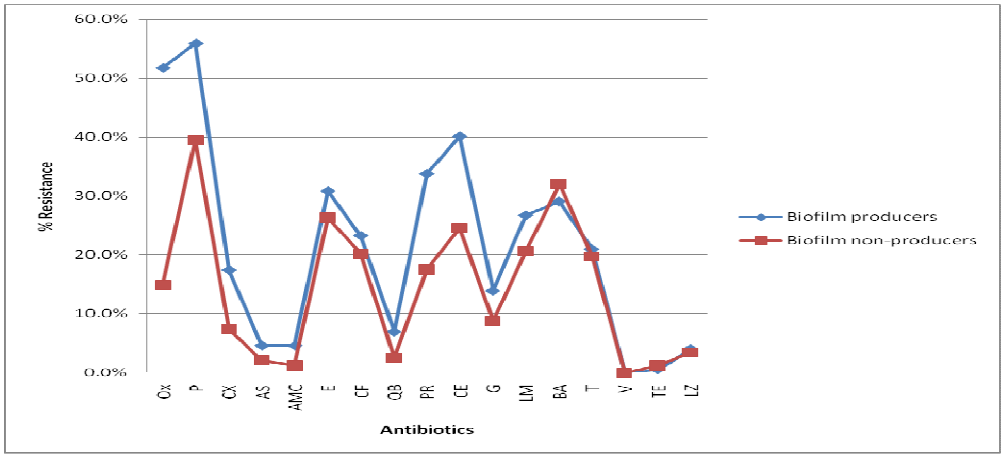

Figure-4
Antibiotic resistance pattern of Biofilm producers and non-producers

A total of 400 clinical isolates of staphylococci were isolated from various clinical specimens which included 250 isolates of *S. aureus* and 150 isolates of (CoNS). 29.2% of *S. aureus* and 33.33% of CoNS were found Methicillin resistant when tested with oxacillin disc diffusion method.

Production of biofilm was detected by three phenotypic methods. (Table 1). Tube method could detect strong biofilm production in 82 (20.5%) isolates of staphylococci which include 36 (43.9%) MRSA, 21 (25.6%) MSSA, 16 (19.5%) MRCoNS and 9 (11.0%) MSCoNS . It could detect moderate biofilm production in 63 (15.8%) isolates of staphylococci whereas 255 (63.8%) isolates were biofilm non producers.

Congo red agar (CRA) method could detect 101 (25.3%) of staphylococci as strong biofilm producers which include 40 (39.6%) MRSA , 28 (27.7%) MSSA, 21 (20.8%) MRCoNS and 12 (11.9%) MSCoNS. Biofilm production was not detected in 299 (74.8%) staphylococci.

Tissue culture plate (TCP) method could detect 105 (26.3%) of staphylococci isolates as strong biofilm producers which include 42 (40.0%) MRSA, 30 (28.6%) MSSA, 23 (21.9%) MRCoNS and 10 (9.5%) MSCoNS . It could detect 68 (17.0%) staphylococci as

moderate biofilm producers and 227 (56.8%) as non-producers of biofilm.

TCP method could detect more biofilm producing isolates. Chi square test showing p value<0.001 indicates that there is significant difference in the results of three tests and TCP method gives significantly more positive results than the other two methods. Mathur et al. (2006) have reported TCP method as gold standard for biofilm detection. Considering this, TCP method was used as standard method and other results were interpreted based on TCP method results.

According to the TCP method, maximum biofilm producing staphylococci were isolated from patients with artificial devices (89.5%) followed by those isolated from blood (45.9%) and pus (42.7%). Only 26.3% of staphylococci isolated from sputum samples were biofilm producers as shown in Table 2.

Amongst various species of staphylococci 45.6% of *S. aureus* isolates and 38.7% of CoNS were biofilm producers. Biofilm production was highest in *S. epidermidis* (53.7%) followed by *S. haemolyticus* (50%) as shown in Table 3.

Biofilm production was detected in 72.36% methicillin resistant and 30.32% methicillin sensitive staphylococci. 80.82% of MRSA and 60% of MRCoNS were biofilm producers as shown in Table 4. Biofilm producers were found to be more resistant to almost all the classes of antibiotics whereas biofilm non producers were comparatively less resistant. (P value-0.04 which is<0.05) All the isolates were sensitive to vancomycin by MIC method. Only 4.1% of biofilm producers were resistant to Linezolid whereas 0.6% were resistant to Teicoplanin. (Graphical representation in Fig. 4)

DISCUSSION

Biofilm formation is reported to be an important characteristic of all staphylococcal species associated with the infection of biomedical devices [11,18,19]. Biofilm production in staphylococci isolated from other clinical samples are also of clinical significance as biofilm constitutes reservoir of pathogens and are associated with resistance to antimicrobial agents and chronic infections. In view of this, a reliable and easy method for their diagnosis is necessary.

We tested 400 clinical isolates of staphylococci by three in-vitro screening procedures for their ability to form biofilm.

TCP method could detect 43.3% of staphylococcal isolates to be biofilm producers which could be further classified as 26.3% strong producers and 17% moderate producers. This method gave the best discrimination between strong, moderate and non-production of biofilm. Bose et al. reported that 54.19% of staphylococci were biofilm producers whereas Mathur et al. reported 53.9% of staphylococci as biofilm producers[20,17]. In our study, 45.6% of *S. aureus*, 38.7% of CoNS and 53.7% of *S. epidermidis* were found to be biofilm producers. Fatima Khan et al. have reported 64.89% of *S. aureus* as biofilm producers[6]. Adilson Oliveira detected 84% CoNS as biofilm producers[21]. Both the findings were higher than our study which might be because of the difference in the sources from which the strains were isolated. We found a very high incidence of biofilm production in staphylococcal isolates from patients with artificial devices (89%) whereas it was only 26.3% in staphylococci isolated from sputum samples. Similar high incidence of biofilm production in patients with artificial devices had also been reported by Mulla and Revdiwala[7] .

The CRA method was found to be easier and faster to perform than other phenotypic methods but could probably identify only the strong biofilm producers. It is imprecise in the identification of moderately biofilm producing strains. Hence, it could detect the least number of biofilm producers.

Tube method could detect less number of strong biofilm producers as compared to other two methods. It could discriminate between strong and moderate biofilm producers .However, the interpretation is observer dependent and there are chances of subjective errors. Further it was hard to differentiate between moderate and non-biofilm producers. This is in agreement with previous reports of Christensen's et al. and Mathur et al.[11,17] and hence cannot be

recommended as a general screening test to identify biofilm producing isolates.

There are some highly accurate methods like PCR analysis to detect ica gene as a virulence marker of biofilm. Aricola et al. and O'Gara and Humphreys have reported that Biofilm non producers are negative for ica A, ica D and lack entire ica ADBC operon[19,22]. However in developing countries like India, a low cost method which requires less expensive equipment and technical expertise is needed. We suggest TCP method based on our findings. It is also reported as gold standard by Mathur et al.[17]. Similar finding have been reported by other researchers[6,20]. Hence TCP method was considered as standard method for further interpretation of results. In our study, 80.82% of MRSA and 60% MRCoNS were biofilm producers. Eiichi Ando et al. have reported a very high percentage (95.4%) of biofilm production in MRSA in Japan[23]. In an Indian study Fatima Khan et al have reported 87.64% of MRSA as biofilm producers[6]. Methicillin resistance of *S. aureus* has been shown to influence biofilm formation[24]. Biofilm producing strains were found to be more resistant to almost all the groups of antibiotics as compared to biofilm non-producing strains. This finding is important because treatment of patients with MRSA infections becomes further more difficult when the strain is biofilm producer, as biofilm is known to impede the delivery of antibiotics[2]. This observation is supported by various other researchers[20,25,26].

CONCLUSION

There is an association between biofilm production with persistent infection and antibiotic therapy failure[27]. Hence identification of infection caused by biofilm producing staphylococci might help modify the antibiotic therapy and prevent infection. TCP method can be adopted as most suitable and reproducible method for detection of such strains.

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