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Research article

DETECTION OF BIOFILM PRODUCTION IN BLOOD CULTURE ISOLATES OF *STAPHYLOCOCCI*

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ABSTRACT

Background: Biofilm producing bacteria which are inherently resistant to antibiotics and disinfectants are widely associated with implant associated infections. *Staphylococcus* is the most commonly associated pathogens with bloodstream infection**. Aims**: The current study was conducted to detect biofilm production in *Staphylococci* isolated from blood culture specimens. **Materials and Methods:** 70 clinically significant staphylococcal isolates from blood culture were screened for biofilm production by Tissue culture plate (TCP) method, Tube method (TM) and Congo red agar (CRA) method and their antibiotic susceptibility profile was studied. **Results:** 59 out of 70 staphylococcal isolates were positive by TCP, out of these 21.4% *staphylococci* were high biofilm producers, 62.8% *staphylococci* were moderate biofilm producers and 15.8% were non-biofilm producers. Maximum resistance was observed in biofilm producers to cotrimoxazole (74.5%) and erythromycin (62.7%) and none were resistant to vancomycin and linezolid. Out of total 59 biofilm producers, 20.3 % (12) were methicillin resistant and all these were *S. aureus* isolates. 19% (1) out of total 11 biofilm non-producers were methicillin resistant. **Conclusion:** Biofilm production was seen to be a major virulence factor in most of the staphylococcal isolates obtained from patients with signs and symptoms of septicaemia. *S. aureus* was found to be the major pathogen and timely detection of biofilm producing phenotype should be carried out using a simple and reproducible method, TCP which is both qualitative and quantitative.

Keywords: Biofilm, Blood culture, *Staphylococci*

INTRODUCTION

Staphylococcus epidermidis and *S. aureus* are common causes of nosocomial infections and infections on indwelling medical devices, which characteristically involve biofilms[1.] *Staphylococcus* is the most commonly associated pathogens with blood stream infection (38%) [2,3]. *S. epidermidis* is a part of the normal bacterial flora of the human skin and mucous membranes and cause infection only after penetration of the skin or mucous membranes, usually by trauma, inoculation, or implantation of medical devices and subsequently causing septicemia or endocarditis .Some strains of *Staphylococcus epidermidis* produce slime, a complete glycoprotein which helps them to colonize foreign bodies like vascular catheters or indwelling prosthesis[4,5]. *S. aureus* biofilm-associated infections are difficult to treat with antibiotics and devices need to be replaced more frequently than those infected with *S. epidermidis*. In addition, they represent a

reservoir of dissemination of *S. aureus* infection to other sites in the human body[6,7].

Research performed has revealed that the production of a biofilm is a two-step process involving an initial attachment and a subsequent maturation phase, which are physiologically different from each other and require phase-specific factors. A final detachment (or dispersal) phase involves the detachment of single cells or cell clusters by various mechanisms and is believed to be crucial for the dissemination of the bacteria, in the case of pathogens to new infection sites in the human body [8].

The present study was undertaken to detect the prevalence of biofilm producer and non producer *Staphylococci* isolated from blood specimen in our laboratory by three different methods, viz. tissue culture plate (TCP) method, tube method (TM) and Congo red agar (CRA) method and to compare the above mentioned three different methods for biofilm detection and to study the profile of antibiotic drug resistance.

MATERIALS AND METHODS

After obtaining Institutional ethical clearance and taking informed written consent, a total of 70 consecutive Staphylococcal isolates obtained from blood cultures of patients with fever (>38 degree Centigrade) chills, tachycardia and attending a tertiary care hospital of Uttarakhand, over a period of twelve months was further analysed. Bacteremia was defined as per CDC/NHSN Surveillance Definitions for Specific Types of Infections, except that even a single blood culture growing CONS was not considered as a contaminant [9]. All clinically significant blood cultures positive for CoNS nosocomial bacteremia were isolated and identified as Staphylococcal species by Gram staining, Catalase and Coagulase tests. Patients on antibiotics were excluded.

Reference strains of Staphylococcus epidermidis ATCC 35984 (formerly RP62A) (biofilm producer) and HAM 892 (non biofilm producer) as positive and negative controls respectively were included in this study.

Detection of biofilm production of 70 Staphylococci species was done by following methods: Reference strains of Staphylococcus epidermidis ATCC 35984 (formerly RP62A) (biofilm producer) and HAM 892 (non biofilm producer) as positive and negative controls were included in this study.

1. Tissue culture plate method (TCP)

The TCP method described by Christensen et al [10] is most widely used and is considered as a standard test for detection of biofilm formation. Isolates were inoculated in Trypticase soya broth (10 ml with 1% glucose) from overnight culture on nutrient agar and incubated at 37 ºC for 24 hours. This was further diluted 1 in 100 with fresh medium. 96 wells flat bottomed tissue culture plates were filled with 0.2 ml of diluted cultures and only sterile broth served as control to check sterility. Similarly control organisms were also diluted, incubated and put in tissue culture plates. The culture plates were then incubated at 37ºC for 24 hours. After incubation, gentle tapping of the plates without inverting was done. The wells were washed with 0.2ml of phosphate buffer saline (pH 7.2) four times to remove free floating bacteria. Then adherent biofilm was fixed with 2% sodium acetate and stained with 0.1% crystal violet. Optical densities (OD) of stained adherent biofilm were obtained with a micro ELISA auto reader (Mindraymorepan MR 96 A) at wavelength 570nm. An experiment was performed in triplicate and it was repeated thrice.

Table 1: Classification of Biofilm formation of Staphylococcal isolates based on OD values obtained from TCP method (n=70)

OD Value of TCP	Adherence	Biofilm Formation
> 0.24	Strong	High
0.12-0.24	Moderate	Moderate
< 0.12	None	None

2.Congo Red Agar Method (CRA)

Freeman et al [11] described this alternative method of biofilm screening. 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 a concentrated aqueous solution and autoclaved at 121ºC for 15 minutes. Then it was added to autoclaved brain heart infusion agar with sucrose when the agar was cooled to 55ºC. Plates were inoculated with test organism and incubated at 37ºC for 24 to 48 hours aerobically. Positive (high) result was indicated by black colonies with a dry crystalline consistency. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated a moderate result and red/ pink colonies showed non biofilm producing isolates

3. Tube Method [12]

Trypticase soya broth (10 ml with 1% glucose) was inoculated with test organism of overnight culture from nutrient agar. The broths were incubated at 37 ºC for 24 hours. The cultures decanted and tubes were washed with phosphate buffer saline (pH 7.3). The tubes were then dried and stained with 0.1% crystal violet. Excess stain was washed with deionized water. Tubes were dried in inverted position. In positive biofilm formation, a visible stained film was seen lining the wall and bottom of the tube. An experiment was done in triplicate for 3 times and read as absent, moderate and strong[1.]

Statistical analysis: Data was analysed by using statistical software (excel and epi-info). The comparative statistical analysis for all methods by using 2X2 table given by Greenhalgh. Data obtained from standard TCP method was considered as gold standard for this study and was thus compared with other two methods. Parameters like sensitivity, specificity, positive predictive value, negative predictive value and accuracy were evaluated. Antibiotic sensitivity testing was conducted of all isolates by Kirby Bauer disc diffusion method

RESULTS

A total of 70 clinically significant isolates of *staphylococci* were obtained. Mean age of the patients was 47.8 years. Maximum number of patients were in the age group of 51-60 years (22.8%) followed by 41-50 years (17.1%). *Staphylococci* were almost equally isolated from both males and females in a ratio of 1.1:1 and maximum number of *staphylococci* were obtained from IPD patients (95.7%). 55.7% of them were from Medicine and allied ward, whereas 7 patients (10%) were from Critical care units. 32.8% patients were suffering from kidney disease followed by fever under evaluation and respiratory diseases (15.8% each). Table 2. shows the comparative detection rates by different methods.

Considering TCP as gold standard, data from CRA and TM were compared. True positives (i.e. biofilm producers) were 59 out of 70 staphylococcal isolates, which were positive by TCP. As per classification of biofilm formation by OD values obtained by TCP method, 21.4% *staphylococci* were high biofilm producers, 62.8% *staphylococci* were moderate biofilm producers and 15.4% were non-biofilm producers. Similar pattern was seen in *S. aureus* and CONS isolates.

Table 2: Comparison of Biofilm detection using Congo red Agar (CRA), Tissue Culture Plate (TCP) and Tube method (TM)

	S.aureus **(n=52)**	**CONS (n=18)**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
TCP(Taken as gold standard for comparison)							
Positive Negative	44(84.6%) 8(15.4%)	15(83.3%) 3(16.7%)					
CRA			72.8%	36.3%	86%	20%	67.2%
Positive Negative	42(80.7%) 10(19.2%)	8(44.5%) 10(55.5%)					
TM			72.8%	72.7%	93.5%	33.4%	72.9%
Positive Negative	35(67.3%) 17(32.6%)	11(61.1%) 7(38.8%)					

PPV-positive predictive value, NPV-Negative predictive value

Table 3: Comparative analysis of biofilm formation by TCP, CRA and TM in different regions of India

	Current study		Mathur et al[1]		Bose et al [2]		Khan et [13]	
	CRA	**TM**	**CRA**	**TM**	**CRA**	**TM**	**CRA**	**TM**
Sensitivity	72.8%	72.8%	6.8%	73.6%	8.25%	76.27%	89.13%	95.78%
Specificity	36.3%	72.7%	90.2%	92.6%	96.34%	97.56%	67.65%	99.40%
PPV	86%	93.5%	66.6%	93.4%	72.72%	97.36%	91.73%	99.11%
NPV	20%	33.4%	25.3%	66.6%	47.02%	77.66%	69.83%	95.29%

Table 4: Association of indwelling devices with Biofilm production

	RISK FACTOR							
	Foley's catheter	%	**Ryle's tube**	%	**CVC/ IV cannula**	%	**Other indwelling devices**	%
Biofilm producers (N=59)	27	45.8%	18	30.5%	59	100%	**06**	**10.2%**
Biofilm non-producers (N=11)	04	36.7%	03	27.3%	08	72.7%	**00**	**0%**
TOTAL	**31**		**21**		**67**		**06**	

Out of total 59 biofilm producers, 20.3 % (12) were methicillin resistant(MR) and all these were *S. aureus*isolates. Out of total 11 biofilm non-producers only 19% (1) were MR. Out of 13 MRSA 92% (12) were Biofilm producers.

In biofilm producing strains of *staphylococci* all patients had Central venous catheter (CVC)/IV cannula, 45.8% patients had Foley's catheter, 30.5% had Ryle's tube and 10.2% had other indwelling devices as shown in table 4. Fifty five patients stayed <10 days and 85.5% of their blood culture isolates showed biofilm production whereas only 3 patients stayed for more than 30 days and all three blood culture isolates showed biofilm production as shown in table 5.All the data was retrieved from patient case records.

Table 5: Association of Duration of hospital stay with biofilm production

Duration of hospital stay (days)	**Number of patients**	**Biofilm producers**	**Percentage**
< 10	55	47	**85.5%**
11-20	8	6	**75%**
21-30	4	3	**75%**
>30	3	3	**100%**
Total	**70**	**59**	

Antibiotic susceptibility of biofilm producers and non producers as per figure 1shows that Vancomycin and linezolid resistance was not detected in any of the isolates. Maximum resistance was observed in biofilm producers to cotrimoxazole (74.5%) and erythromycin (62.7%). Due to few numbers it was not possible to state whether this difference was statistically significant as shown in fig 1.

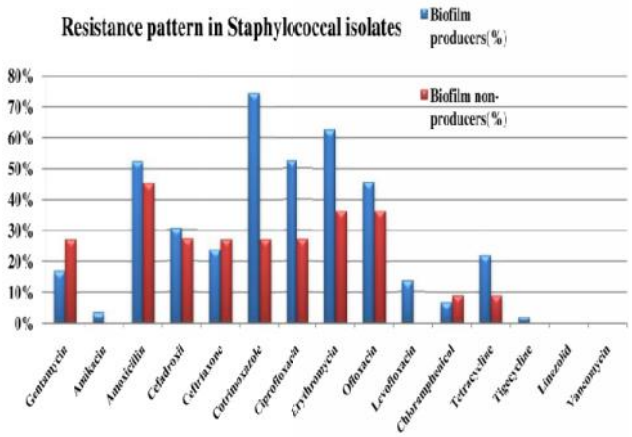

Fig 1: Resistance pattern in Staphylococcal isolates

Maximum number of patients i.e. 46 (65.7%) recovered, whereas 13 (18.6%) expired. No difference was noted in the outcome of patients infected with or without biofilm producing *staphylococci*.

DISCUSSION

Biofilm formation is an important characteristic of all staphylococcal species, associated with the infection

of biomedical devices [13]. National institute of Health report that more than 60% of infections in health care are caused by biofilms[2.] A total of 70 clinically relevant staphylococcal isolates were obtained from blood cultures during the study period. 52 (74%) were *S. aureus* and 18 (26%) were CONS. *A*lthough the formation of biofilm on indwelling medical devices is generally associated with CONS, *S.aureus* strains are also capable of production of biofilm[2]. In our study maximum blood culture isolates were *S. aureus.* Biofilm production is considered to be a marker of clinically relevant infection caused by *S. aureus* and isolation of *S. aureus* from blood culture represents true infection and isolation of CONS as a contaminant[14,15], however recent studies recommend that even single isolation of CONS from patients with clinical signs of sepsis[16]. In the current study similar isolation rates of biofilm production was seen in *S. aureus* and CONS.

Although usefulness of species identification of CONS in clinical laboratory has not met with universal agreement, most microbiologists and clinicians recommend the need to identify them. It is generally recommended now to report isolates as CONS if speciation is not being done [9], like in our case.

Biofilm production by *staphylococci* have been evaluated mainly by TCP, the gold standard method, and CRA & TM, which are simple and inexpensive tests, but results have been found to be variable in different studies conducted so far as shown in Table 2.

In our study we had a higher biofilm detection rate by modified TCP (84.2%) as compared to other workers using traditional TCP method[1,2,13.] Modified TCP has been taken as a gold standard as it has been recommended as superior to TCP by several researchers [2,13,17-19]. Furthermore higher detection rate of biofilm in our study can be attributed to the fact that our study isolates were obtained from clinically relevant cases of bacteremia[9], a majority who had pre-existing indwelling devices like CVC, Intravenous cannula, Foley's catheter, Ryle's tube and Endotracheal tube which predispose to developing bacteremia by biofilm producing strains of *S. aureus* and CONS. Similarly high detection rates of biofilm formation was reported by Khan F et al [13] (64.89%) as compared to other workers like Mathur et al [1] (53.8%)and Bose et al [2] (54.19%) who had studied biofilm production in isolates obtained from other clinical specimens too including blood.

We found better specificity, PPV, NPV with TM as compared to CRA especially in the biofilm producing strains, same has been observed in TCP and TM by various researchers [1,2,13,20].

Staphylococcal infections with biofilm production are extremely difficult to eradicate and antibiotic treatment may not give desired clinical benefits .In these cases invasive treatments like removal of infected device and surgical removal of infected tissue may be necessitated. Hence timely detection of biofilm producing phenotype should be carried out using TCP method in patients with hospital acquired infections and also in Methicillin resistant staphylococcal infections [21] .Various studies recommend the use of a combination of detection methods especially for blood culture isolates of *staphylococci*. Grinholc and co-workers [22] showed that among 48 *ica*genes positive *S. aureus*isolates from bacteremia, 50% and 46% produced biofilm on CRA and TCP, respectively. Lorio et al [19] found a similar rate of positivity for both CRA (67.5%) and TCP (62.5%) in 40 *S. aureus* isolates from blood cultures that were positive for the *ica* gene and this figure increased to 85% (P =0.022 in relation to TCP and P =0.066 in relation to CRA) when the results of both phenotypic methods were combined, making the correlation with the presence of the *ica* gene closer. Moreover, since negative isolates for the *ica* gene were also negative for both phenotypic methods analyzed, they suggest that a combination of methods would more accurately predict the presence of this gene in *S. aureus* isolates from blood cultures. Among the studies that have employed the two phenotypic methods one detected 100% positivity by both methods in 44 *ica* genes positive *S. epidermidis* isolates from blood[23]and Lorio et al [19]found detection rates of 66.7% for TCP and 41.7% for CRA increasing to 75% when they used a combination of both methods for *ica* gene positive *S.epidermidis* isolates.

Our results were similar to those described by these authors, out of 70 staphylococcal isolates 84.2% were positive by TCP and 74.4% by CRA and 94.3% (66/70) were positive by CRA and TCP thus showing that these isolates were clinically relevant.

Bacterial colonization of CVC's occurs rapidly and biofilm can be found on the CVC's of all patients

whose catheter had been in place for less than 3 days and bacteria can adhere to medical devices as early as within 24 hours. Catheters in place for 10 days tend to have extensive biofilm formation on the external surface of the catheters. For long-term catheters (up to 30 days), biofilms are more extensive on the internal lumen [24,25].

In our study the antibiotic resistance pattern of biofilm producing *staphylococci* was higher than in biofilm non producers and the same has been reported by other workers too[2,26,27]. It was noted that MR strains of *staphylococci* (92%) were more prone to biofilm formation as compared to the methicillin sensitive strains of *staphylococci* (82.5%). Similarly biofilm producers were more MR (20.3%) when compared to biofilm non producers (9%). Methicillin susceptibility of *S. aureus* has been shown to influence the biofilm formation [9].

It was seen that out of follow up available of 49 patients; 20.4% of patients infected with biofilm producing staphylococcal strains had expired and 30% of patients infected with non-biofilm producing strains had expired. No statistical difference could be observed in these groups. Other coexisting morbid conditions of the patients may have been responsible for the patient's outcome.

Limitation of study : We have only carried out phenotypic tests for detecting biofilm production and detection of ica gene was not done furthermore speciation of CoNS would have provided a better picture of clinical relevance and effectiveness of the methods carried out for detection of biofilm production.

CONCLUSION

Biofilm production was seen to in most of the staphylococcal isolates obtained from patients with signs and symptoms of septicaemia. *S. aureus* was found to be the major pathogen. Biofilm production was detected equally in both *S. aureus* and CONS. Using TCP 84.2% of *Staphyloccocci* from blood cultures were detected with biofilm production Since these infections are extremely difficult to eradicate timely detection of biofilm producing phenotype should be carried out using a simple and reproducible method like TCP .

It is recommended that more reliable methods for detecting biofilm producers should be developed and preventive strategies be worked out to prevent their production since this will reduce infection rates and their associated morbidity**.**

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Conflict of Interest: None

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