Original Article

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Microbiology Section

Study of Biofilm Formation Among Clinical Staphylococcal Isolates

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ABSTRACT

Introduction: Biofilm forming microorganisms are related to chronic and recurrent human infections as well as are highly resistant to antimicrobial agents. Various methods have been used in medical field for the detection of biofilm production which includes visual assessment by electron microscopy and polymerase chain reaction, quantitative method like Tissue Culture Plate (TCP) method and two qualitative methods such as Tube Method (TM) and Congo Red Agar (CRA) method.

Objectives: To detect the prevalence of biofilm formation in *Staphylococci*, to evaluate two different methods i.e. TM and CRA for the detection of biofilms and to see its relation with antimicrobial resistance.

Materials and Method: The study was conducted at Department of Microbiology, Dr. D.Y. Patil Hospital and Research Centre during the period June 2011 to June 2013.

A total of 130 clinical isolates were subjected to biofilm detection methods. Standard microbiological procedure was done to identify the isolates. Biofilm detection was tested by TM and CRA. Kirby-Bauer disc diffusion technique was performed to do antibiotic susceptibility test of biofilm producing bacteria according to CLSI guidelines.

Results: From the total of 130 clinical isolates, biofilm productions among *Staphylococci* were 59.23%. And the percentage of biofilm detection of *Staphylococci* isolates by Congo red method were 31.54% and by test tube method 50%. Hence, the TM method was *cons*idered to be superior to CRA. We have also observed higher antibiotic resistance in biofilm producing bacteria than non-biofilm producers.

Conclusion: We can conclude from our study that the TM method is a more suitable and reliable method as compared to CRA and can be recommended as a general screening method in laboratories for detecting biofilm forming bacteria.

Keywords: Antimicrobial resistance, Congo red agar method, Test tube method

INTRODUCTION

Staphylococci being recognised as important cause of disease around the world are of particular concern because of offering resistance to a wide range of antibiotics. In today's world infection holds serious *cons*equences due to additive biofilm forming ability of multi drug resistant strains [1]. Biofilms are common form of microbial growth characterised by the cells enclosed in self produced extracellular polymeric substances (EPS) irreversibly attached to each other or to substratum [2].

Biofilm production is *cons*idered as a marker of clinically relevant infection. The identification and information on the biofilm producing strains would help a clinician in assessment of its virulence as well as plan proper treatment for patients [3]. The objective of this study was evaluation and comparison of biofilm forming ability of *Staphylococci* by two conventional qualitative methods namely TM and the CRA method where TM was taken as standard.

MATERIALS AND METHODS

This was an experimental study conducted at Department of Microbiology, Dr. D.Y. Patil Hospital and Research Centre, Kolhapur, Maharashtra during the period June 2011 to June 2013.

Inclusion Criteria

- When gram stain of the smear from the specimen showed predominantly gram positive cocci in clusters along with pus cells.
- Strains isolated in pure culture from specimen.

Exclusion Criteria

- Organism isolated as mixed growth.
- CoNS isolates with no clinical correlation with symptoms.

After obtaining ethical clearance, a total of 130 (Staphylococcal isolates) from clinical samples- which includes pus, blood, urine, catheter tip, sputum, endotracheal tube, throat swab, pleural fluid, vaginal swab and ascitic fluid were processed in the department of Microbiology, during the study period within two hours of receipt as per standard procedures. Direct microscopy of gram stain smear for various morphological

types of organisms and presence or absence of pus cells was done.

Specimens were inoculated in Nutrient agar, Blood agar and MacConkey agar plates. The plates were incubated aerobically at 37°C overnight and observed for the growth, colony morphology and pigment production after 24 hours. All the suspected colonies are examined microscopically after gram staining for presumptive identification of organism according to their Gram's reaction, morphology along with Catalase, modified oxidase, Oxidation-fermentation, Furazolidone sensitivity and Bacitracin sensitivity tests. Speciation into *Staphylococcus aureus* and *CoNS* was done by different biochemical tests including Coagulase test (slide coagulase test and tube coagulase test), Mannitol salt agar test, Alkaline phosphatase test, Urease test ,Voges proskauer test, Carbohydrate fermentation test and Novobiocin sensitivity test. Coagulase test is usually used to distinguish among *Staphylococcus* spp. into two groups Coagulase positive *Staphylococci* (*Staphylococcus aureus*) and Coagulase negative *Staphylococci* [4].Then detection of biofilm Formation was done by Congo Red Agar and Tube Method

Congo Red Agar Method: CRA medium is proposed by Freeman et al., [5].

Preparation of CRA medium:

- Contents of CRA medium : BHI agar-37gms/l
- Sucrose- 50 gms/l
- Agar no.1- l0 gms/l
- Congo red stain- 0.8 gms/1

Concentrated aqueous solution of congo red stain was prepared first then autoclaved at 121°C for 15 minutes separately. Further it was added to autoclaved BHI agar with sucrose at 55°C and then poured in 90 mm petri plates. The medium is inoculated with test *Staphylococci* and incubated at 37°C for 24 to 48 hours aerobically [5].

Interpretation:

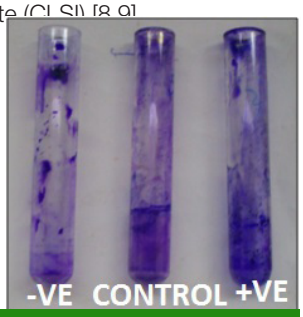
- **Biofilm producers:** Black colonies with a dry crystalline *cons*istency [Table/Fig-1].
- **Non biofilm producer:** Pink colonies [Table/Fig-1]

Tube Method: Biofilm production was investigated by the tube adherence test proposed by Christensen et al., [6].

From slope culture of strain to be tested, loopful of colonies were inoculated in 10 ml Trypticase soy broth with 1% glucose and incubated at 37^{0}C for 24 hours under aerobic condition. The tube content were decanted after 24hrs, washed with phosphate buffer saline (pH 7.3) and dried. After complete drying tube was stained with 0.1% crystal violet. De-ionized water was used to remove excess stain. Tubes were again dried in inverted position [6]. The presence of a layer of stained material adhered to the inner wall of the tubes is *cons*idered as positive. The exclusive observation of a stained ring at the liquid-air interface was not *cons*idered to be positive result [6] [Table/Fig-2].

Reference strain of positive biofilm producer *Staphylococcus epidermidis* ATCC 35984, *Staphylococcus aureus* ATCC 35556 and *Staphylococcus epidermidis* ATCC12228 (non-slime producer) were used as control [7].

Antimicrobial sensitivity testing: Antimicrobial sensitivity testing was done by Kirby- Bauer disc diffusion method for the following antimicrobial agents: Penicillin 10 units/disc, Erythromycin 15µg/disc, Clindamycin 10µg/disc, Vancomycin 30 µg/disc, Gentamycin 10 µg/disc, Cotrimoxazole 25 µg/disc, Ampicillin 10 µg/disc, Tetracycline 30 µg/disc, Nitrofurantoin 300 µg/disc .The strength of discs used and their zone size interpretative standards were according to norms of Clinical and Laboratory Standard Institute (CLSI) [8,9].



[Table/Fig-1]: Congo red method for biofilm detection
[Table/Fig-2]: Test tube method for biofilm detection

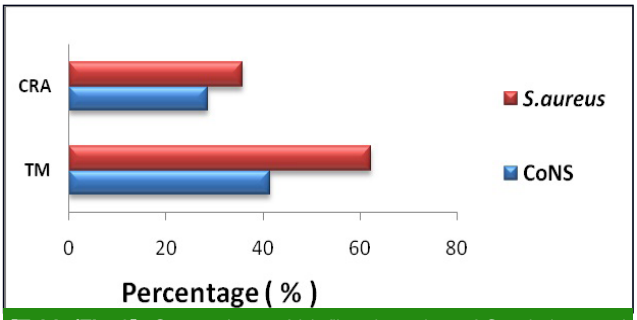
RESULTS

This study was based on 130 Staphylococcal isolates which were isolated from various clinical samples. Majority of isolates of *Staphylococci* were obtained from pus (39.23%) followed by urine (28.46%), Catheter tip (13.06%) and Blood (6.92%).

Biofilm production among *Staphylococci* were 59.23% .

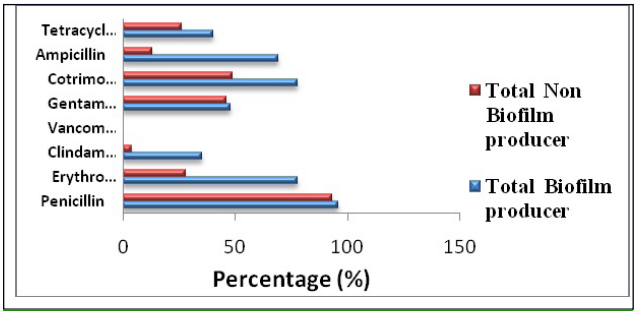
23.08% of Staphylococcal strain were Congo red and tube test both positive, 40.77% were Congo red and tube test both negative, 27.69% were Congo red negative and tube test positive, 8.46% were Congo red positive and tube test negative.

Test tube method detected 62.26% and 41.56% of *S.aureus* and *CoNS* respectively whereas Congo red test detected 35.85% and 28.57% of *S.aureus* and *CoNS* respectively. The percentage of biofilm detection of *Staphylococci* isolates in the present study by Congo red method was 31.54 % and by test tube method was 50 % [Table/Fig-3]



[Table/Fig-3]: Comparison of biofilm detection of Staphylococcal isolates by 2 conventional methods

Antimicrobial susceptibility profile in relation to biofilm production: It was found that biofilm producing strains were resistant when compared to the biofilm non-producers. The resistance pattern of biofilm producing strains when compared to biofilm non producer was for Penicillin 96.1/92.5%, Erythromycin 77.9/28.3%, Clindamycin 35.1/3.8%, Gentamycin 48.1/45.3%, Cotrimoxazole 77.9/49.1%, Ampicillin 68.8/13.2%, Tetracycline 40.2/26.4%. However, all strains were sensitive to vancomycin [Table/ Fig-4].



[Table/Fig-4]: Antimicrobial resistance pattern amongst biofilm producing *Staphylococci*

DISCUSSION

Staphylococcal species associated with biomedical devices infection are reported to have an important biofilm forming characteristics. Other clinical samples isolated biofilm producing *Staphylococci* are also of clinical significance as one of the most confounding aspects of bacterial biofilm formation is its high recalcitrant to antibiotic treatment, which holds serious *cons*equences for therapy of infections that involve biofilms.

The biofilm antibiotic resistance is by following methods, decreased diffusion of antibiotics through the biofilm matrix, decreased oxygen and nutrient availability accompanied by decreased growth rate and expression of resistance genes [10]. Various methods can be used for biofilm detection and in this study we evaluated 130 Staphylococcal isolates by two qualitative screening methods for their ability to form biofilm.

In the present study [Table/Fig-5] *S.aureus* biofilm formation rate were 67.92% and the comparable pattern of biofilm formation by *S.aureus* species was observed by Ammendolia et al., Bose et al,. and Samant et al., while *CoNS* biofilm formation rate were 53.24% which is in correlation with the studies of Cunha MLR et al., Boynukara et al., and Bose et al.,[Table/Fig-6] [11-15]. The findings showed variation to our study which might be because of the difference in the sources from which the strains were isolated.

The percentage of biofilm detection of *Staphylococci* isolates in the present study by CRA method were 31.54% and by TM 50% which is similar to study done by S.sharvari et al.,. (25%) and V. Purthi et al., (50%) respectively[13,16]. Another study done by T.Mathur et al., and Bose et al., reported lower percentage as compared to our study [17,12]. In contrast Fatima et al., reported higher percentage of biofilm detection amongst *Staphylococci* by both methods [Table/Fig-7][18].

Study series	Year	Test tube	Congo red
V Pruthi et al.,[16]	2003	50.5%	61.1%
T Mathur et al.,[17]	2005	41.44%	5.2%
Bose et al.,[12]	2009	42.46%	6.15%
Fatima et al.,[18]	2011	63.74%	47.79%
S sharvari et al., [13]	2012	36.3%	25.3%
Present study	2013	50%	31.54%

[Table/Fig-7]: Percentage of biofilm detection amongst Staphylococcal isolates by two methods in various studies

Detection of ica genes as a virulence marker of biofilm can be done by some highly accurate methods like PCR analysis. Aricola et al., and O'Gara have reported that biofilm non producers lack entire ica ADBC operon and are negative for ica A, ica D [19,20]. However, in developing countries like India, a low cost method requiring less expensive equipment and technical expertise is much needed. Hence, we suggest TM method based on our findings as CRA method is imprecise in the identification of moderately biofilm producing strains. Hence, it could detect the least number of biofilm producers in comparison to TM method.

The resistant pattern of biofilm producers to antibiotics were higher in comparison to non biofilm producers. The increased antibiotics resistance of biofilm producing strains may be due to their slow rate of metabolism and infrequent division resulting in decreased sensitivity to antibiotics targeted at cellular functions such as protein and DNA synthesis. (lewis,2007) [21]. This observation is supported by various other researchers also [10,12].

Studies	Ammendolia et al., (19999)[11]		Bose et al., (2009)[12]		S.sharvari et al., (2012) [13]		Present study	
S.aureus	No.of strains	Biofilm +ve	No.of strains	Biofilm +ve	No.of strains	Biofim +ve	No.of strains	Biofilm +ve
	63	88.8%	68	32.96%	250	46%	53	67.92%

[Table/Fig-5]: Biofilm formation by *S.aureus* in various studies

Studies	Cunha MLR et al.,(2004)[14]		Boynukara et al.,(2007) [15]		Bose et al.,(2009)[12]		Present study	
CoNS	No.of strains	Biofilm +ve	No.of strains	Biofilm +ve	No.of strains	Biofilm +ve	No.of strains	Biofilm +ve
	51	17.6%	65	60%	111	45%	77	53.24%

[Table/Fig-6]: Biofilm formation by *CoNS* in various studies

CONCLUSION

Biofilm production is associated with persistent infections and antibiotic therapy failure thereby posing a major challenge for the physicians along with economic relevance as well. Hence, such problems can be prevented by detection of biofilm producers and appropriate antibiotic doses modification. Therefore, test tube method can be adopted as most suitable and reproducible method for detecting such strains. However, discrimination between strong and moderate biofilm producers are not possible with tube method and the interpretations are observer dependent that are subjective to errors .

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