RESEARCH PAPER | Microbiology | Volume : 5 | Issue : 4 | April 2015 | ISSN - 2249-555X

Detection of Biofilm Formation Among the Clinical Isolates of Staphylococcus Aureus

KEYWORDS: Biofilm, Staphylococcus aureus, Tissue Culture Plate method, Tube method.

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ABSTRACT *BACKGROUND: Biofilms cause significant problems in the environment and during the treatment of infections. Biofilm-associated infections are characteristically chronic and frequently occur in hospitals. Staphylococcus aureusis a leading cause of such infections [1].*

OBJECTIVE: The objective of this study was to detect the biofilm-forming ability of Clinical isolates of S. aureus in our Hospital.Material andMethods:Among 90 Staphylococcus spp., only 45 (50.00%) strains were identified as S. aureus by standard conventional microbiological methods. These strains were screened by Tissue Culture Plate (TCP), & Tube method (TM) for detection of biofilm formation.Results: Of 45 (50.00 %) strains of S. aureus, 01 (20.00 %) in Blood samples, 2 (20.00 %) in Pus, 03(42.85 %) in Urine, 02 (33.33 %) in Sputum, 05 (50.00%) in Urinary catheter tips &06 (85.71%) in Central venous catheters. Strong biofilm production was detected in TCP method 11 (57.89%) as while Moderate biofilm producers by TM method detected is 08 (42.10%).Antibiotic susceptibility testof biofilm producing bacteria was performed by using the Kirby-Bauer disc diffusion technique,&higher antibiotic resistance was observed in biofilm producing bacteria than non biofilm producers. Conclusion: We can conclude from our study that the TCP method was found to be most Sensitive, accurate &reliable method for the detection of biofilm forming microorganisms as compared to TM, and it can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

INTRODUCTION:-

Microorganisms universally attach to surfaces and produce extracellular polysaccharides, resulting in the formation of a biofilm. Biofilms pose a serious problem for public health because of the increased resistance of biofilm-associated organisms to antimicrobial agents and the potential for these organisms to cause infections in patients with indwelling medical devices [2]. All microbes like Gram positive and Gram negative bacteria have capacity to synthesize biofilm. Bacteria commonly involved include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Enterococcus faecalisKlebsiellapneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa*.[3]

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 extracellular polysaccharide called Polysaccharide Intercellular Adhesin (PIA). PIA is involved in cell to cell adhesion and is essential for biofilm production [4].Biofilm formation is regulated by expression of polysaccharide intracellular adhesin (PIA), which mediates cell to cell adhesion and is the gene product of *icaADBC*.Various reports attest to the presence of *icaADBC*gene in *S. aureus* and *S. epidermidis* isolated from infections associated with indwelling medical devices[5].

Staphylococci are most often associated with chronic infections of implanted medical devices. Such infections are predominately caused by *Staphylococcus aureus* and *Staphylococcus epidermidis*. The biofilms protect the cells not only from host immune response but also from antimicrobial agents. Indeed, biofilm formation is a major concern in nosocomial infections because it protects microorganisms from opsonophagocytosis and anti-biotics, leading to chronic infection and sepsis.These qualities have converged to make *S. aureus* a significant burden on our current health care system. One of the patient populations most vulnerable to *Staphylococcus aureus* infection are those with implanted medical devices such as central venous catheters, cardiac valves and pace-makers, artificial joints and various orthopedic devices. Therefore, once biofilm-associated *S. aureus* infections occur, they are difficult to be treated by conventional procedures [6].

The use of indwelling medical devices is important in the treatment of critically and chronically ill patients, however bacterialcolonization of implanted foreign material can cause major medical and economic sequel.It is now well documented that biofilms are notoriously difficult to eradicate and are often resistant to systemicantibiotic therapy and removal of infected device becomes necessary. The differentiation of staphylococci with respect to its biofilm phenotype might help to elucidate the impact of staphylococci in diagnosis of infections related to biomedical devices and these observations may have utility in the prevention of device related infections[5]

While there are many techniques available for biofilm study, it is imperative that standardized techniques should be developed. A variety of methods have been standardized in various laboratories, each having their own merits. The Standared methods include tissue culture plate (TCP), tube method (TM) & Congo red agar method (CRA)[7,8]. The objective of this study was to detect the formation of biofilm by *S. aureus* isolated from different clinical speci-

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mens by two conventional methods, namely tissue culture plate (TCP) , tube method (TM).

AIMS AND OBJECTIVES

The present study was undertaken

- To detect biofilm production by *S. aureus* in our hospital and
- To evaluate two differentmethods i.e. Tube Method (TM) and Tissue Culture

Plate Method (TCP) for their detection and

- To assess the relation of biofilm formationwith Antimicrobial resistance.

MATERIALS AND METHODS

Selection of Samples, Place and duration of the study: -After obtaining institutional ethical clearance, a total of 90 non-repetitive clinical isolates of *Staphylococcus* spp.wereisolated from Blood,pus, urine, sputum,Urinary catheter tips &Central Venous catheters from both In patients & Out patients were processed in department of Microbiology at Aarupadaiveedu medical college, Pondicherry,during a period of six months i.e., January 2014 to June 2014.

***Staphylococcus aureus* isolates**

Out of 90 *Staphylococcus* spp, 45 strains were identified as *S. aureus* on the basis of standard and conventional microbiological techniques including Colony morphology,Gram stain, catalase,slide and tube coagulase tests & these 45 isolates were subjected to biofilm detection methods, and antibiotic susceptibility test by Kirby Bauer's disc diffusion method on Mueller Hinton (MH) agar and the zones were interpreted as per CLSI guidelines[4]

Detection of biofilm formation:

Biofilm detection was done by two methods:

(1) Tissue culture plate (TCP)&

(2)Tube method (TM)

Tissue culture plate method (TCP)

The TCP assay described by Christensen *et al*[7]is most widely used and was considered as standard test for detection of biofilm formation. In present study, we screened all isolates for their ability to form biofilm by TCP method as described by Christensen *et al*[7] with a modification in duration of incubation which was extended to 24 hours. Previous reports have indicated the influence of media composition on biofilm production, therefore we had evaluated biofilm production in three different media, tryticase soy broth (TSB Difco), TSB with 1% glucose (TSBglu) and brain heart infusion (BHI, Difco) with 2% sucrose (BHISuc)[5]

Isolates from fresh agar plates were inoculated in respective media and incubated for 18 hour at 37°C in stationary condition and diluted 1in100 with fresh medium. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates (Tarson, Kolkata, India) wells were filled with 0.2 ml aliquots of the diluted cultures and only broth served as control to check sterility and non-specific binding of media.

The tissue culture plates were incubated for 18 hours and 24 hours at 37°C. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 mL of phosphate buffer saline (PBS pH 7.2) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent staphylococcal cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria were determined with a micro ELISA auto reader (model 680, Bio rad) at wavelength of 570 nm (OD570 nm). These OD values were considered as an index of bacteria adhering to surface and forming biofilms.

Experiment was performed in triplicate and repeated three times, the data was then averaged and standard deviation was calculated. To compensate for background absorbence, OD readings from sterile medium, fixative and dye were averaged and subtracted from all test values. The mean OD value obtained from media control well was deducted from all the test OD values. [5]

Classification of bacterial adherence

For the purpose of data calculation, we used classification (Table 1) based on OD values obtained for individual strainsof *Staphylococcus* spp[7]

Table 1: Classification of bacterial adherence by TCP method

Mean OD values	Adherence	Biofilm formation
<0.120	Non Moderately Strong	Non / weak Moderate
0.120-0.240		
>0.240		High

Tube method (TM)

A qualitative assessment of bifilm formation was determined as previously described by Christensen *et al*.[8] TSB-glu (10mL) was inoculated with loopful of microorganism from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with PBS (pH 7.3) and dried.dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation.

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate or 3-strong Experiments were performed in triplicate and repeated three times[5]

RESULTS:

√ Out of 90 clinical samples of *Staphylococcus* spp, 45 (50.00%) strains were identified as *S. aureus*, 19 (42.22%) showed biofilm positive production by two conventional methods., Tissue culture method & Tube method.

√ Among 45 (50.00 %) strains of *S. aureus* isolated from various clinical samples, (1) 20.00 % of Bio film production was observed in Blood samples, (2) 20.00 % in Pus, (3) 42.85 % in Urine, (2) 33.33 % in Sputum, (6) 85.71% in Central Venous catheters & (5) 50.00% in Urinary catheter tips, Shown in **Table 2**.

√ Comparison of biofilm production by clinical isolates of *S. aureus* by two conventional methods is given in **Table 3.** Out of 45 clinical samples of *S. aureus*, 11 (57.89%) showed biofilm positive production by Tissue culture plate method & 08 (42.10%) by Tube method.

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- √ Screening of 45 *S.aureus* isolates for biofilm formation by TCP method in different media and at 18 and 24 hours of incubation **Table 4**
- √ In the standard TCP assay, only 2 biofilm positive isolates in 18 hrs& 03 in 24 hrs were detected of 45 tested in TSB medium, whereas with addition of 1% glucose in TSB glu, number of biofilm forming isolates increased from 05 in 18 hrs to 07 in 24 hrs and Similarly, 03 in 18 hrs& 06 after 24 hours of incubation respectively, by using BHIsuc medium biofilm forming isolates were detected **Table 4.**
- √ In modified TCP method, from the total number of 45 isolates tested for biofilm formation, strong biofilm producers were 7 (15.55%) by TSB glu, 08 (17.77%) were moderate by TSB and 05 (11.11.0%) isolates byBHIsuc and 34 (75.55%)were considered as non or weak biofilm producers **Table 4.**
- √ The TM showed good correlation with the TCP assay for strongly biofilm forming isolates i.e., among total 08 (17.78%) isolates 05 (11.11%) were picked up as strong and 03 (6.66%) were moderat biofilm producers.
- √ Antibiotic susceptibility test of biofilm producing bacteria was performed by using the Kirby-Bauer disc diffusion technique, & higher antibiotic resistance was observed in biofilm producing bacteria than non biofilm producers. **Table 5**

DISCUSSION:

Bacterial biofilms are estimated to play a major role in more than 80% of bacterial infections.[9,10] Sixty percent of hospital-associated infections are ascribed to the formation of biofilms on implantable medical devices.[11] In addition, there are many chronic and refractory diseases associated with biofilms, such as native valve endocarditis, cystic fibrosis pneumonia, periodontitis, chronic rhinosinusitis, and otitis media.[12,13]

Indwelling medical devices are frequently used in all health setup while critical care units of hospitals use multiple medical devices for treatment and intervention in patient care. [14]

Implant infections still remain the major complication in clinical use of the biomaterials, therefore it is obvious that new therapeutic and preventive strategies have to be developed and introduced. During the long evolutionary journey, microorganisms had enough time to master their mechanisms allowing them to adhere and persist on practically every type of surface. As was mentioned, there is presently no completely "biofilm-proof" biomaterial. It is known that even the use of the antibiotic-loaded biomaterials, which seemed to be a very promising strategy, needs significant improvements. The main challenges that have to be solved are the kinetics of antibiotics in the biomaterials, possible alteration of the physicochemical structure and, often observed, a decrease of the biomaterial biocompatibility in result of antibiotic incorporation [15].

Slime production has been reported in strains of all *Staphylococcus* spp. associated with the infection of biomedical devices.Investigations to understand the pathogenesis of these infections have focused upon the process of adherence of these microorganisms on these devices. Investigators have used various methods to quantify number of microorganisms adhering to surfaces[5]

Biofilm and multidrug resistance have been identified as virulence factors of great magnitude in *Staphylococcus aureus* infections in clinical settings. *S. aureus* is a medically important organism associated with a vast variety of diseases; some strains can cause chronic infections and gain increased resistance to antimicrobial agents through biofilm formation [16,17].Researchers have investigated the strategies employed by microorganisms to produce biofilms and to understand the pathogenesis. They discovered that biofilm-producing bacteria secrete certain chemicals that protect them from disinfectants and antimicrobials, and phagocytic host immune systems [17].

Out of 90 clinical samples of *Staphylococcus* spp, 45 (50.00%) strains were identified as *S. aureus*, 19 (42.22%) showed biofilm positive production by two conventional methods., Tissue culture method & Tube method.Among 45 (50.00 %) strains of *S. aureus* isolated from various clinical samples, Central venous catheters (CVCs) showed high biofilm production (6) 85.71% in Central Venous catheters than (5) 50.00% in Urinary catheter tips.

In our study, out of 45 *Staphylococcus aureus* isolates 11 (57.89%) showed biofilm positive production by Tissue culture plate method & 08 (42.10%) by Tube method.TCP showed 11 (57.89%) positive biofilm production using TCP method when we used TSB, TSBglu&BHIsuc. Similar reports have been given by other studies when using TSB as a medium and extending the incubation time to 24 hours [5].

In the TCP assay with TSB medium, only 08 (17.77%) of 45 tested S.*aureus*isolates displayed a biofilm positive phenotype. This was in agreement with observations of other investigators in which only few or no biofilm producing isolates could be detected using this medium.[18]On the other hand supplementation of TSB and BHI media with different sugars such as glucose and sucrose exhibited biofilm formation in 7 (15.55%) and 05 (11.11.0%) isolates respectively.

In modified TCP method, extended incubation for 24 hour could lead to a better discrimination between moderate and non-biofilm producing S.*aureus* and biofilm formation was observed in11 (57.89%) isolates. These observations suggested a strong dependence between growth condition and biofilm formation in staphylococci and that the use of various sugar supplementations is essential for biofilm formation[18]

The tube test correlates well with the TCP test for strongly biofilm producing isolates but it was difficult to discriminated between weak and biofilm negative isolates due to the variability in observed results by different observers. Consequently, high variability was observed and classification in biofilm positive and negative was difficult by tube method. In agreement with the previous reports, tube test cannot be recommended as general screening test to identify biofilmproducing isolate[8].

CONCLUSION:

Biofilm formation can cause a multitude of problems in the medical field, particularly with prosthetic devices such as indwelling catheters and endo-tracheal tubes. There is an association between biofilm production with persistent infection and antibiotic therapy failure[19]. Hence identification of infection caused by biofilm producing staphylococci might help modify the antibiotic therapy and prevent infection. Due to the high level of morbidity and mortality as well as the high frequency of infection associated with *S. aureus*, represents an important potential clinical target for the prevention of chronic infections associated with prosthetic medical devices.Obtaining clinical samples

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from such devices for laboratory testing to identify biofilm formation can help prevent potentially fatal and persistent infections.

We can conclude from our study that the TCP method was found to be most Sensitive, accurate & reliable method for the detection of biofilm forming microorganisms as compared to TM, and it can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

RESULTS- TABLES

Table 2. Biofilm production of 45 *Staphylococcus. aureus* strains examined according to the source of isolation.

Source	*S. aureus* (n = 45)				Total
	Biofilm +	(%)	Biofilm -	(%)	
BLOOD	01	20.00	04	80.00	05
PUS	02	20.00	08	80.00	10
SPUTUM	02	33.33	04	66.66	06
URINE	03	42.85	04	57.14	07
CENTRAL VENOUSCATHETERS	06	85.71	01	14.28	07
URINARY CATHETERS	05	50.00	05	50.00	10
TOTAL	19	42.22	26	57.77	45

Table 3. Comparison of the production of biofilm by clinical isolates of *Staphylococcus aureus* by two conventional methods

Methods	*Biofilm Positive S. aureus* (n=19)	
	Biofilm +	%
Tissue Culture Plate	11	57.89
Tube Method	08	42.10

Table 4: Screening of 45 *S.aureus* isolates for biofilm formation by TCP method in different media and at 18 and 24 hours of incubation

Biofilm formation (OD) 570nm	No. of isolates					
	TSB		TSBglu		BHI suc	
	Incubation period (hour)					
	18	**24**	**18**	**24**	**18**	**24**
High (> 0.240 ± 0.022)	**02**	**03**	**05**	**07**	**03**	**06**
Moderate (0.120-0.240 ± 0.020)	09	08	06	04	08	05
Weak/ Non (< 0.12 0 ± 0.012)	11	11	11	11	11	11

Table 5: Resistance pattern of *S.aureus* isolates (n=45)

Antibiotic Sensitivity Testing of S.aureus n=45			
S.NO	Name of Antibiotic	Biofilm producing organisms % n=19	Non-biofilm producing organisms % n=26
1	Ampicillin	45	80
2	Ciprofloxacin	56	81
3	Clindamycin	95	100
4	Co-trimoxazole	50	77
5	Erythromycin	46	61
5	Gentamycin	54	77
6	Linezolid	30	50
7	Oxacillin	60	78
8	Tetracycline	45	75
9	Ticarcillinclavulanic acid	70	90
10	Tigecycline	60	86
11	Teicoplanin	80	98
12	Vancomycin	90	100

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