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Comparison of Different Methods for Detection of Biofilm Formation in *Staphylococcus aureus* in a Tertiary Care Hospital

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ABSTRACT

Biofilm is the population of bacterial cells growing on the biotic and abiotic surfaces embedded in a matrix of extracellular polysaccharide, which facilitate the adherence of microorganism. This study is aimed to compare and evaluate different Methods for detection of Biofilm formation in *Staphylococcus aureus* isolates. For comparison of different methods of Biofilm production, 100 isolates of *Staphylococcus aureus* (50 MRSA and 50 MSSA) were taken and screened by tissue culture plate method, tube method in different condition and Congo Red Agar (CRA) plate method. Out of 100 isolates of *Staphylococcus aureus*, 19% shows Strong biofilm formation, while 33 % showed moderate and 48 % isolates showed weak/No biofilm production by TCP Methods. In TM-Gr-1: 10%, 28% and 62%; in TM-Gr-2: 10%, 20% and 70%; in TM-Gr-3: 16, 29% and 55% isolates showed strong, moderate and weak/ No biofilm production respectively. The CRA Method showed 17% biofilm production. The TCP method was found to be most sensitive screening method for detection of biofilm formation by staphylococcus aureu. MRSA isolates were more Biofilm producers than MSSA.

Introduction

Biofilm is the population of bacterial cells growing on the biotic and abiotic surfaces embedded in a matrix of extracellular polysaccharide, which facilitate the adherence of microorganism. *Staphylococci* are most common organism associated with chronic infections of implanted medical devices (Astha and Amita Jain, 2012; Donlan, 2002).

The use of indwelling medical devices in clinical set up is important in the patient management in Intensive care unit as well as of critically and chronically ill patients, however bacterial colonization of implanted foreign material can cause major medical and economic sequel.

The increased use of indwelling medical devices has increased the impact of *Staphylococci* in clinical settings. The predominant species isolated in these infections are *Staphylococcus aureus* and *Staphylococcus epidermidis*. Biofilm formation is their major pathogenicity (Kloos *et al.*, 1994).

Biofilm is a group of organisms growing as a layer on a surface. Biofilm thickness can range from a single cell layer to a substantial community encased by a viscous polymeric milieu. Structural analyses have shown that in some cases unique pillar or mushroom-shaped structures can be formed by the micro-colony architecture of these dense biofilms; however, other structures do form depending on the environmental conditions (Costerton *et al.,* 1995). Biofilm consists of multilayered cell clusters embedded in a matrix of extracellular polysaccharide, which facilitates the adherence of these microorganisms to biomedical surfaces or devices and protect them from host defence system and antimicrobial treatment (Gara *et al.,* 2001).

Staphylococcus aureus is well-known bacteria to make biofilms on different human body surfaces as well as indwelling devices. The microbes forming the biofilm are difficult to treat in clinical settings. These isolates may or may not be resistant to anti-bacterial agents in laboratory setting, but due to difficulty in eradication of the biofilm formed on the surfaces of the devices/appliances and protection provided to the microorganism by protective covering of adhesive biomaterial (slime), it becomes difficult to treat infections caused by these organisms. An association between antibiotic resistance and biofilm production in nasal isolates of *Staphylococci* have been reported.

Astha and Amita Jain (2012) found in their study that there is a significant high rate of Biofilm production in nasal isolates of *S. aureus*. Various researchers (Astha, 2012; katayama *et al.,* 2000) have observed that Antibiotic resistance among biofilm producer was significantly higher than that of Non-biofilm producers.

Staphylococci are ubiquitous pathogens that usually produce biofilms during different infectious processes, which are generally difficult to treat. It has been estimated that about 65 per cent of the hospital acquired infections are associated with biofilm forming organism. It has been assumed that these infections are 10 to 1000 times more difficult to eliminate with an otherwise successful treatment. The mechanism for enhanced antimicrobial resistance is believed to involve alteration in gene expression leading to a phenotypic difference between the planktonic and sessile forms. The planktonic forms are more resistant as they produce exo-polysaccharide, have different growth characteristics and take up nutrients and drugs differently as compared to their planktonic counterparts.

De Araujo *et al.,* (2006) reported that biofilm producing Methicillin resistant *S. epidermidis* isolates from healthy individuals from the community had a higher incidence of multi-resistance than biofilm non-producers from the same population. They also noticed increased incidence of multi resistance among biofilm producers compared to non-producers. It is mentioned clearly in literature that over 65% of hospital based infections caused by the biofilm producing organisms (Kloos and Bannerman, 1997). It is now well documented that biofilm are notoriously difficult to eradicate and are often resistant to systemic antibiotic therapy and removal of infected device becomes necessary (Lewis, 2001; Schwank *et al.,* 1998; Souli *et al.,* 1998; Astha and Amita Jain, 2012).

The detection of biofilm producer and non producer *Staphylococci* 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 and better patient management (Astha and Amita Jain, 2012). There are number of phenotypic tests available to detect biofilm production by *Staphylococci* which

include tissue culture plate (TCP), tube method (TM), Congo red agar (CRA) (Christensen *et al.,* 1985; Freeman, 1989 and Mathur *et al.,* 2006).

Materials and Methods

A total of 100 non-repetitive, clinical and carriage isolates of *Staphylococcus* species were investigated for the production of Biofilm by using different methods. Isolates were initially identified by standard microbiological techniques including Gram stain, catalase test, coagulase test, Mannitol fermentation and DNase test.

All cultures were maintained on Brain Heart Infusion agar (Himedia Ind Pvt Ltd.). Known reference strains of *S. epidermidis* ATCC 35984 (high slime producer), *S. epidermidis* ATCC 35983 (moderate slime producer) and *S. epidermidis* ATCC 12228 (non slime producer) obtained from Himedia Ind Pvt Ltd. were used as controls in the study (Winn *et al.,* 2006; Mackie and McCartney practical medical microbiology. 14th ed, 2012; Koneman, 2016 and CLSI, 2017)

For comparison of different methods of Biofilm production, 100 isolates of *Staphylococcus aureus* (50 MRSA and 50 MSSA) were taken and screened by Tissue Culture plate Method (TCP) by using BHI broth supplemented with 2% sucrose for 24 hrs.

Three different conditions was applied in Tube Method (TM) by incubating isolates at 370C in BHI broth supplemented with 1% Glucose for 24 hrs (Group-1), and in BHI broth supplemented with 1% sucrose for 24 hrs (Group-2) and in BHI broth supplemented with 1% sucrose for 48 hrs (Group-3). Congo Red Agar (CRA) Plate method was also used to detect biofilm production.

Tissue culture plate method (TCP)

The TCP assay described by Christensen *et al.,* (1985) and Mathur et al (2006) was used in this study. For comparative study of different methods TCP method was considered as standard test for detection of biofilm formation. In this study, we had screened all isolates to check their ability to form biofilm by TCP method as described by Christensen *et al.,* (1985) with a modification in duration of incubation which was extended to 24 hours.

Isolates from fresh agar plates were inoculated in Brain heart infusion broth media supplemented with 2% sucrose and incubated for 18 hour at 37oC 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 slime producing organisms in the tissue culture 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. Slime forming adherent staphylococcal cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density (OD) of the tissue culture plate was calculated by micro ELISA auto reader at wavelength of 570 nm (OD- 570 nm). The observed OD values were considered as an index of bacteria adhering to surface and biofilm formation.

To compensate for background absorbance, the OD value of sterile medium is subtracted from the OD of all test values. Thus, the mean OD value obtained from media control well was deducted from all the test OD values which give the Result.

Interpretation of the result

The optical density will be recorded using an ELISA reader at the wavelength of 570 nm. The strains that gave an OD value less than 0.120 will be recorded as non-biofilm producers, those with OD values between 0.120 and 0.240 will be considered as moderate producers and those with OD values more than 0.240 will be considered as strong producers.

Tube method

A qualitative assay for biofilm formation was determined as previously described by Christensen et al (1982) with some modifications in media introduced and incubation time.

Three different conditions was applied in Tube Method (TM) by incubating isolates at 370C in BHI broth supplemented with 1% Glucose for 24 hrs (Group-1) and in BHI broth supplemented with 1% sucrose for 24 hrs (Group-2) and in BHI broth supplemented with 1% sucrose for 48 hrs (Group 3).

Each group of Liquid culture media (10ml) was inoculated with loopful of microorganism from overnight culture plates and incubated for 24 hours and 48 hours at 37°C. The tubes were decanted and washed with Phosphate Buffer Saline (pH 7.3), dried and then stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionised water. Tubes were than dried in inverted position and observed for a visible film of the stain.

Biofilm formation was considered as positive when a visible layer of crystal violet smeared the wall and bottom of the tube. Ring formation at the liquid interface is indicative of non biofilm formation. Tubes were observed for the intensity of colour adherence and the amount of biofilm formation was recorded as 0-absent, 1-weak, 2-moderate or 3-strong.

Congo red Agar method (CRA)

The bio film detection by Congo Red Agar was performed as per the method described by Mathur *et al.,* (2006) for detection of biofilm a special media was prepared. The medium was composed of BHI (37 gms/L), sucrose (100 gms/L), agar no.1 (10 gms/L) and Congo red stain (0.8 gms/L). Congo red was prepared as concentrated aqueous solution by autoclaving at 121°C for 15 minutes. It was prepared separately from other medium constituents and then added to the agar when cooled to 55°C. Plates were poured make let them allow to settle for about 10-15 minutes. Then the isolates were inoculate and incubated aerobically for 24 to 48 hours at 37°C.

Interpretation of the results

Positive result was indicated by black colonies with a crystalline dry consistency. Weak producers usually remained red. A darkening of the colonies without the dry crystalline colonial morphology indicated an indeterminate result.

Results and Discussion

Out of 100 isolates of *Staphylococcus aureus*, 19% (14 MRSA and 05 MSSA) shows Strong biofilm formation, while 33 % showed moderate (21 MRSA and 12 MSSA) and 48 % isolates (15 MRSA and 33 MSSA) showed weak/No biofilm production by Tissue Culture Plate Methods. In Tube Method-Group-1,

10% of *S. aureus* isolates show strong biofilm producer in which 7 were MRSA and 03 MSSA were MSSA. Similarly 28% of isolates showed moderate biofilm producer among which 20 isolates were MRSA and 08 were MSSA while 62% isolates were non biofilm producer among which 23 isolates were MRSA and 39 isolates were MSSA); in Tube Method-Group-2, 10% (7 MRSA and 03 MSSA), 20% (12 MRSA and 08 MSSA) and 70% (31 MRSA and 39 MSSA); in Tube Method-Group-3, 16% (12 MRSA and 04 MSSA), 29% (18 MRSA and 11 MSSA) and 55% (20 MRSA and 35 MSSA); isolates showed strong, moderate and weak/ No biofilm production respectively. The CRA Method showed least and only 17% of isolates to be biofilm producer among which 12 isolates were MRSA and 5 were MSSA. Biofilm production with highly correlated with TCP method and Tube Method Group-3 but was least sensitive method.

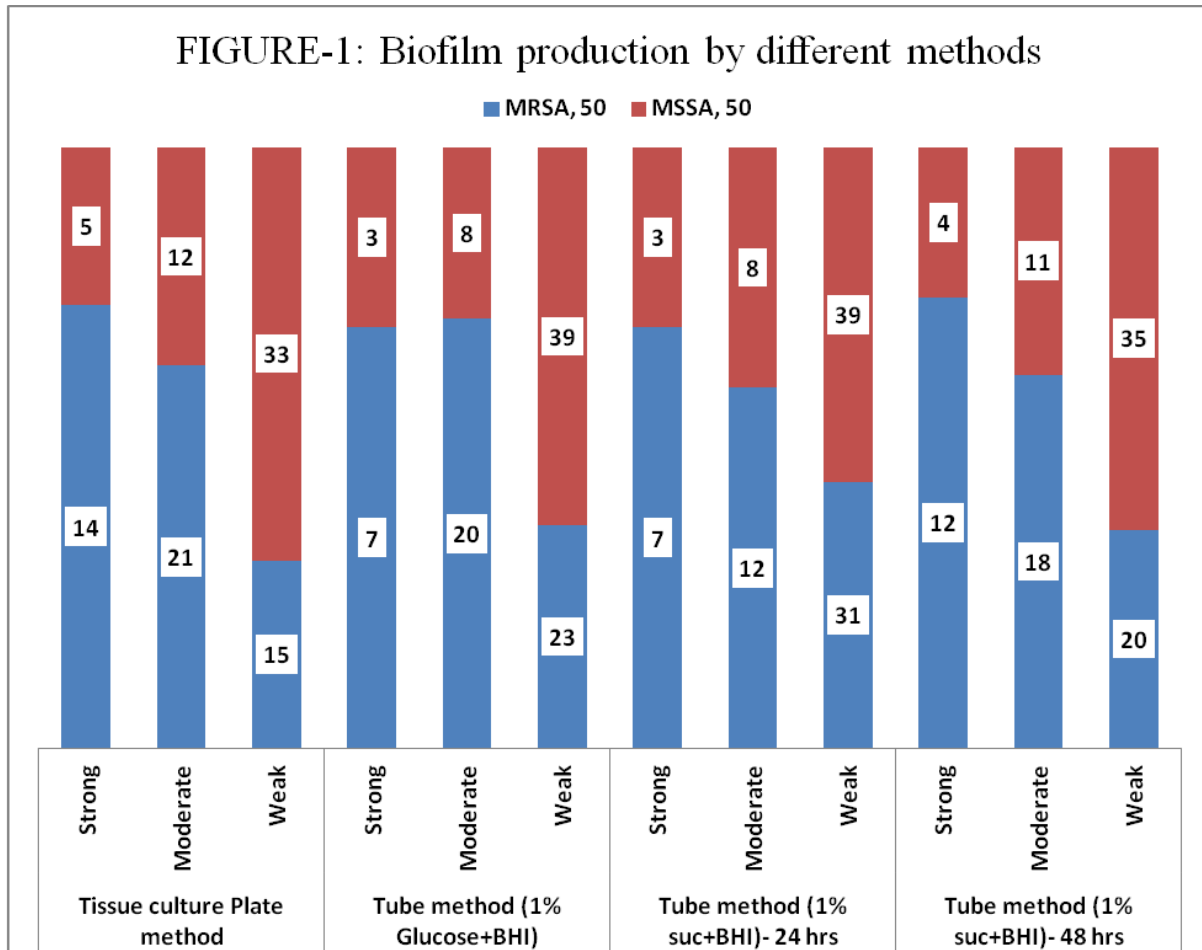
The TCP method found to be the most effective screening method for detection of biofilm formation by *Staphylococcus aureus* and has the advantage of quantitative analysis of the biofilm formation by *Staphylococci*. MRSA isolates were more Biofilm producers than MSSA.

Biofilm production has been reported in strains of all *Staphylococcus* species associated with the infection of biomedical devices (Kooles *et al.,* 1994). 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 (Panda *et al.,* 2014; Donlan, 2001) We tested 100 clinical and carriage isolates of *Staphylococci* by three in vitro screening procedures to detect the ability of biofilm production.

In the Tissue culture plate method, by using BHI broth supplemented with sucrose; only 52 % *Staphylococcus* isolates show biofilm production. In modified TCP method, incubation period is extended for 24 hour. It provides a better discrimination between moderate and non-biofilm producing *Staphylococci* and biofilm formation.

While correlating with test tube method with the TCP test method for strong biofilm producing isolates, it was difficult to discriminated between weak and biofilm negative isolates due to the variability in observed results by different observers which was also experienced by other authors (Sasirekha *et al.,* 2012; Maryam Rezaei, 2013) Consequently, high variability was observed and classification in biofilm producer and Non Producer was difficult by tube method. This depends upon skill of the researcher. In agreement with the previous reports, tube test cannot be recommended as general screening test to identify biofilm-producing isolate (Christensen *et al.,* 1982). In CRA method, out of 100 staphylococcal isolates, seventeen (17%) showed black colonies with dry crystalline morphology. These observations are entirely in disagreement with observations reported by the Maryam Rezaei *et al.,* (2013) and A Jain *et al.,* (2012) in which he found better sensitivity of the method. The similar finding was observed by Praharaj *et al.,* (2013), Tayal Ruche (2015) which established that the TCP method is more reliable, sensitive and specific (Fig. 1).

Based on our results and observation us contempt to the CRA method for detection of biofilm formation for *Staphylococcus* isolates. Our data indicates that the TCP method is an accurate and reliable method for screening and this technique can also able to serve as a reliable quantitative tool for determining biofilm formation.



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