

Detection of Biofilm development by *Staphylococcus aureus* and its role in antibiotic susceptibility pattern.

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Abstract

Staphylococcus aureus is a causative agent of many types of infections throughout the world. This infections are of major concern because of the causative agent offering resistance to a wide range of commonly used antibiotics. *Staphylococcus aureus* which is gram +ve , Coagulase +ve, non spore forming bacteria is also responsible for the biofilm production. Biofilm constitute reservoir of pathogens and are associated with resistance to antimicrobial agents and chronic infections. In the present study 180 non duplicated clinical strains of *S. aureus* were screened by Tissue Culture Plate Method and Congo red Agar method. Antibiotic susceptibility of all these strains was carried out by Kirby bauer disc diffusion method. Tissue Culture Plate method 32% were highly biofilm producing and 46 % were moderately biofilm producing. Rest was non biofilm producing by tissue culture plate method. 38% were highly biofilm producing and 51% were moderately biofilm producing remaining 21% were non biofilm producing in case of congo red agar method.

Key Words: antibacterial resistance, biofilm, congo red agar method. *Staphylococcus aureus*, tissue culture plate method.

Introduction

Staphylococcus aureus is the causative agent of many types of infections which leads the simple to fatal types of diseases. This organism is responsible for the production of biofilm. Biofilm formation is a serious more chronic condition that varies accordingly to changes in environment, nutrients and characteristics of the pathogen and the host immune system[4],[18]. Biofilm formation is one of the causative factor for hospital infections by *S. aureus* as *S. aureus* is usually found in the normal flora of healthy human host, the colonization of these organism is a risk factor for the development of disease which directly or indirectly related to the formation of biofilm[5]. In number of studies it was seen that the formation of the biofilm is the major cause for the antibiotic resistance pattern. Antibiotic sensitivity test is carried out by Kirby Bauer Disc Diffusion Method[3].

An infectious process in which biofilm have been implicated include problems such as urinary tract infections, catheter infection, middle ear infection, formation of dental plaque, gingivitis, coating

on contact lens and less common but more lethal process such as infective endocarditic, cystic fibrosis and infection of permanent undwelling devices such as joint prosthesis, and heart valves.

The present study was undertaken to detect the prevalence of biofilm production in *S. aureus* for the period of one year. Tissue Culture Plate Method and Congo Red Method were used for the detection of biofilm and to see its relation with antimicrobial resistance.

Materials and Methods.

The study was undertaken from July 2016 to June 2017. In this study total 180 non duplicated isolates of *Staphylococci* were taken. The isolates were collected from clinical specimens like blood, infected devices, skin surfaces, urine, pus, catheter, respiratory mucus etc. from the hospitalized patients from tertiary care hospital of Nagpur city. An isolated organism was tested and confirmed by Gram Staining, Catalase test and Coagulase test. Three standard reference strains of *Staphylococcus aureus* are also used in present study. These are ATCC 35984 (High Biofilm/Slime Producer), ATCC 35983 (Moderate Biofilm/Slime Producer), ATCC 12228 (as Non Biofilm/Non Slime Producer). For this study the two specifically designed tests were used. The first is Tissue Culture Plate Method and Congo Red Agar Test.

1. Tissue culture plate (TCP) method[8,10]
2. Congo red agar (CRA) method [1,10]

Detection of biofilm producing *staphylococci* was the main aim of this work. The following two methods were used for this purpose

Tissue culture plate method : Loop full test organism from overnight grown culture was inoculated in 10 ml of Trypticase Soy Broth along with 1% of glucose and incubated for 24 hrs at 37° C. The 10 ml of inoculated broth is then diluted again with fresh medium of Trypticase Soy Broth [8,10]. A flat bottom tissue culture plate is filled with this diluted broth by adding 0.2 ml in each of 96 wells in tissue culture plate. After the incubation of 24 hrs at 37°C the broth medium was collected just by gentle tapping on the back of the tissue culture plate. For the removal of free floating bacteria this tissue culture plate were washed for three to four times by 0.2 ml phosphate buffer and saline at pH7.2 [8,10].

After washing three to four times biofilms which remained adherent to the walls and the bottom of the wells were fixed with 2% sodium acetate and then stained with 0.2 % crystal violet. Excess of stain was washed with di-ionized water and plates were allowed to dry properly.

Optical density (OD) of stained adherent biofilm were obtained with a micro ELISA auto reader at wavelength of 570 nm . Experiment was performed in triplicate and repeated thrice. Average (OD) values of sterile medium were calculated and subtract from test value [8,10]. All the steps was also carried out for the control strains used in present study and sterile medium act as a control.

Congo Red Agar Test: In Congo Red Agar test, Brain Heart Infusion broth is prepared (37gm/lit),Sucrose (5 gm/lit)and congo red dye (0.8gms/lit).Congo red dye was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes. Then it was added to the sterilized brain heart infusion media aseptically[1,10]. Test organism is inoculated and incubated for 24to 48hrs at 37°C aerobically. Black colonies with dry crystalline consistency indicated the formation of Biofilm.

The test organisms were parallel processed for the antibiotic susceptibility by Kirby Bauer Disc Diffusion Test. Test was carried out on Muller Hinton Agar (MHA) by using following antibiotic discs. Penicillin(10 μ g), Ampicillin(30 μ g), Ofloxacin(30 μ g),Ciprofloxacin(30 μ g), Cefotaxime(30 μ g),Erthromycin(30 μ g),Cotrimoxazol(30 μ g),Amikacin(30 μ g),Gentamycin(30 μ g),Linez oide(30 μ g),Netilmicin(30 μ g),and Vancomycin(30 μ g).All the antibiotic disc were procured from Hi-Media Pvt. Ltd. India. After Incubation the standard zones were measured and noted for the study of susceptibility pattern[4].

Results: In this present study 180 *S. aureus* were isolated from clinical samples namely blood, pus, catheters, dialysis fluid, CAPD(continuous peritoneal ambulatory dialysis), nasal swab, throat, etc.. While studying the antibiotic susceptibility pattern of various biofilm producers and non biofilm producers it was observed that biofilm producers show more resistance towards antibiotics as compared to non biofilm producers. The observation was also supported by the Mathur et.al[8,10].

All isolates were isolated by standard procedures.[11].The standard procedure includes two different screening tests for biofilm production namely TCP & CRA method. The formation of biofilm on indwelling medical devices is generally associated with the co-agulase negative *S. aureus*. OD value of stained adherent biofilm was obtained with the micro Elisa plate auto reader. The OD were taken at 570nm from the standard readings it was decided that value ≤ 0.120 was considered as non biofilm producing, as moderate biofilm producing and ≥ 0.240 was as highly biofilm producing. In tissue culture plate method 32%(58)samples were highly slime /biofilm producing and 46% i.e.(82)samples were moderately slime producing. About 22% i.e (40) samples were found to be non biofilm producing.

Mean ODValue	Adherence	Biofilm Formation
≤ 0.120	Non	Non/Weak
0.120-0.240	Moderate	Moderate
≥ 0.240	High	High

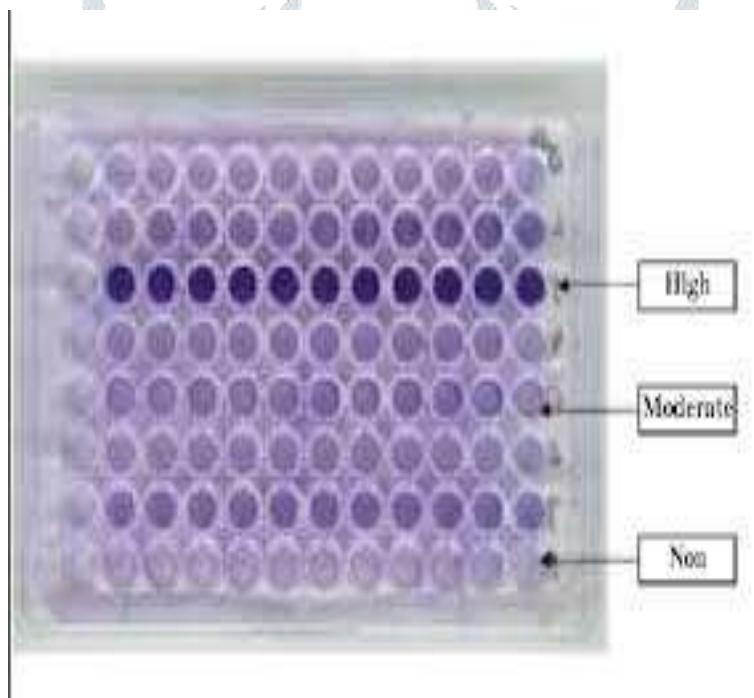
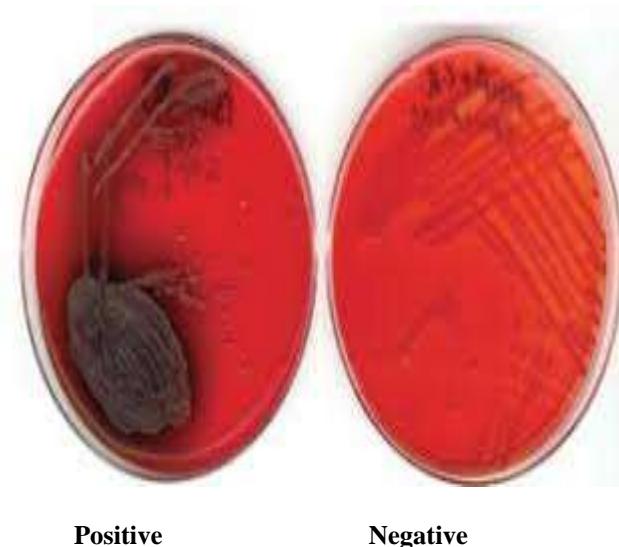
Table:4 Classification depending on OD values obtained from *Staphylococcus aureus* by TCP method.Fig No 1.Antibiotic Susceptibility test *S.aureus* showing susceptible test plate and resistant plate

Fig No 2 Tissue Culture Plate Method for Biofilm producers and Non Biofilm

Producers by *S.aureus*

**Fig No 3 Congo Red Agar test****Table No :1**

Sr.no	Source	No.of Samples	S.aureus		
			Tissue culture plate method		
			Highly +ve	Moderately +ve	Non Biofilm Producing
1	Catheters	35	14	10	11
2	Blood	30	11	12	07
3	Orthopedic implants	45	12	26	07
4	CAPD	30	08	16	06
5	Nasal/Throat/Ear Swab	25	07	12	06
6	Wound	15	06	06	03
7	Total	180	58	82	40
8	Percentage	100%	32%	46%	22%

Table 1: Biofilm production of Staphylococcus aureus with respect to the clinical non duplicated isolates using Tissue Culture Plate Method.

In case of Congo Agar Red test after the incubation period 24 hrs black coloured dry crystalline growth was obtained to get the positive result. Amongst total 180 isolates test, it was found that 38%(68)samples were highly biofilm producing,51%(92)samples were moderately biofilm producing and 11%(20) samples were non biofilm producing. Maximum number of biofilm productions were seen in orthopedic implants in both of the test, followed by samples obtained from catheters and CAPD (continuous peritoneal ambulatory dialysis). Only 11 % (20)samples were found to be non / weak biofilm forming.

Table No.2

Sr.no	Source	No.of Samples	S.aureus		
			Congo Red Agar method		
			Highly +ve	Moderately +ve	Non Biofilm Producing

1	Catheters	35	13	20	2
2	Blood	30	10	17	3
3	Orthopedic Implants	45	16	26	3
4	CAPD	30	13	12	5
5	Nasal/Throat/Ear Swab	25	9	11	5
6	Wounds	15	7	6	2
7	Total	180	68	92	20
8	Percentage	100%	38%	51%	11%

Table No.2:Bilfilm production of *Staphylococcus aureus* with respect to the non duplicated isolates using Congo Red Agar Method.

With respect to the antibiotic susceptibility pattern it was observed that maximum strains showed the resistance. When compared the rate of resistance and biofilm formation it was seen that high resistance to antibiotics, high in biofilm formation. Maximum resistance was seen in penicillin (52%), ampicillin (52%), erythromycin (48%), gentamycin (45%), ofloxacin (43%)and netilmicin(51%) respectively. Linezolid and Vancomycin shows highest sensitivity but not more than 59% and 60%, which is followed by ofloxacin 57%.Rest all the antibiotics were mostly resistant to given antibiotics and responsible for biofilm formation.

Org/Anti	Peni	Amp	Oflox	Cipro	Cefta	Erthy	Co-tri	Amk	Gen	Net	Line	Van
Sen in %	48	48	57	53	52	52	51	53	55	49	59	60
Res in %	52	52	43	47	48	48	49	47	45	51	41	40

Table.No.3 Antibiotic Resistance Pattern among Biofilm Producers as Compared with Biofilm Non Producers.

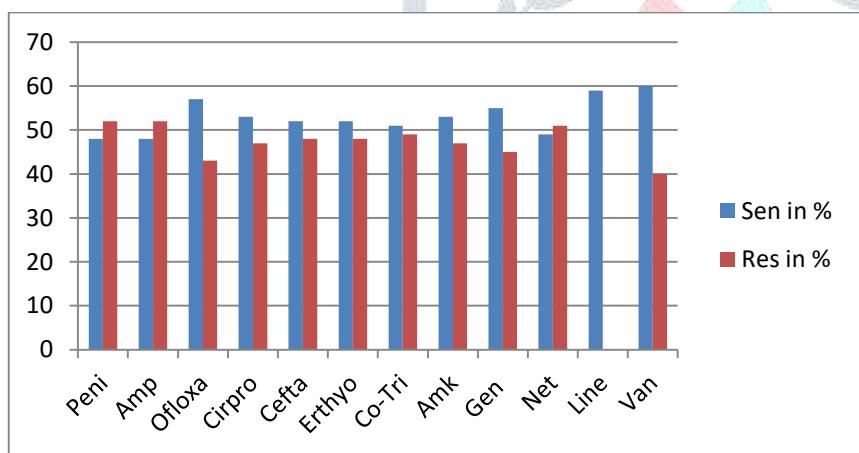


Fig.No.3 Resistance pattern among Biofilm Producers in Comparison with Biofilm Non Producers.

Discussion: Formation of biofilm has long been considered as a virulence factor responsible contributing to infection associated with various medical devices and also causing nosocomial infection [1,5]. Actual process for the biofilm formation cannot be explained but suggested mechanism may be

- a)Detachment of cells from medical device ,biofilm causing bloodstream or urinary tract infection,
- b)Formation of any endotoxin,
- c)Resistance to host immune system,

d) generation of resistance through plasmid exchange[7].

We had total of 180 *Staphylococcus aureus* namely from blood, pus, catheters, nasal, ear, throat swab etc. All were isolated by standard procedures and tested by two in vitro screening methods. **Tissue Culture Plate Method and Congo Red Agar Method.** It was generally observed that Biofilm formation is the characteristic of Co-agulase negative *Staphylococci spp*, but *S.aureus* being Co-agulase positive also shows the formation of biofilm on indwelling medical devices, which was also observed by others too[7,17]. In this antibiotic sensitivity test biofilm producers and non biofilm producers shows significant and clinically relevant observations i.e. higher resistance to conventional antibiotics than non biofilm producers. This same observation was also supported by many workers[8,10]. Only Linezolid and Vancomycin are two antibiotics show sensitivity to more than 50% of the samples rest show an average of 40 to 50%.

Invitro screening test for TCP for biofilm production shows 32% highest +ve, 46% moderately +ve showing similarity to Mathur *et.al*[10]. In CRA test where 38% were highest +ve and 51% were moderately +ve for biofilm production. This test shows highest rate for biofilm formation which is higher than Mathur *et.al*[10]. Our study shows TCP is the better screening test for biofilm production than CRA. The test is easy to perform and assess both qualitatively and quantitatively. In our study, positivity rate of CRA method was higher than observed by other workers, e.g. Mathur *et. al.*[4,10]. From all above discussion we can conclude that, biofilm can be composed of a single or multiple organisms on various biotic and abiotic surfaces. There is association between biofilm production with persistent infection and antibiotic failure. Hence, an infection caused by biofilm producing *staphylococci*, the differentiation with respect to its biofilm phenotype might help to modify the antibiotic therapy and to prevent infection related to biomedical devices. A suitable and reproducible method is necessary for screening of biofilm producers in any healthcare setup and this TCP method can be recommended.

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(Table/Fig 5) Detection of biofilm producers by TCP method. High, moderate and non slime producers were shown on tissue culture plate.

