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A Study on Phenotypic Methods for the Detection of Biofilm Production in Indwelling Medical Devices Used In Intensive Care Units in a Tertiary Care Hospital

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A B S T R A C T

Biofilms are surface associated bacterial communities surrounded by a matrix of exopolymers. Biofilms contribute to the development of persistent infections due to their refractiveness to antibiotic therapy. The purpose of this study was to evaluate three methods for detection of biofilm formation in different indwelling devices. For detection of biofilm formation, out of the 100 indwelling devices processed, 52 bacterial isolates showed growth and these were subjected for biofilm production detection by tissue culture plate (TCP) method, Tube method (TM) and Congo red agar (CRA) method. Of the 52 bacterial isolates, 42 isolates (80.7%) were found to be biofilm producers. The present study revealed that maximum number of biofilm producers, were isolated from the endotracheal tubes (E.T.T.'s) (86%). The most common organism producing biofilm from the E.T.T.'s was *Klebsiella pneumoniae* (36%). Biofilm producers from the intravenous catheter tips (I.V.C.) were 80%, out of which *S. epidermidis* (45%) was found to form highest number of biofilm producers. *Klebsiella pneumoniae* (25%) produced biofilm from Nasogastric tubes (N.G.T.) and 20% of *Acinetobacter baumanii* produced biofilms in Inter Costal Drain tubes (I.C.D.). Out of the 52 isolates subjected for biofilm Production, T.C.P. Method detected 42 (80.7%), T.M. Method detected 38 (66%), C.R.A. Method detected 8 (14%) of the biofilm Producers. Out of the 42 isolates which produced biofilms by T.C.P. method, 10 (23%) were strong, 26 (62%) were moderate & 6 (14%) were weak biofilm producers. Out of the 38 isolates which produced biofilms by T.M., 4(10.5%) were strong, 28 (66%) were moderate, 8 (19%) were weak biofilm Producers. Among the different microorganisms, the frequency of biofilm production showed by 42% of *P. aeruginosa* isolates, 37.5% of *A. baumanii*, 25% *E. coli*, 14% of *K. pneumoniae*. In our study we found that TCPM was the gold standard phenotypic method used for detection of biofilms in resource limited settings. Our study did not recommend C.R.A. method for screening of biofilms due poor sensitivity

Introduction

Biofilms, which are defined as sessile communities of microbes cause a variety of infections. They are characterized by the cells that get irreversibly attached to a substratum or with each other they are embedded in the matrix of Extra Polymeric Substances (E.P.S.)

which they have produced within themselves. Biofilms express an altered Phenotype with respect to growth rate and gene transcription and the Biofilm formation is initiated in response to specific environmental conditions (Donlan and Costerton, 2002). Biofilms are responsible for many device associated infections, dental plaque, upper respiratory tract infections, and urogenital infections, chronic wounds (Reid, 1999).

Biofilms contribute to a serious public health problem because of the increased resistance offered by these organisms to the antimicrobial agents and the potential for them to cause infections in patients with the indwelling medical devices (Sangitharevdiwala *et al.,* 2012).

Medical devices, used frequently in critically ill patients are vulnerable to colonization by the biofilm producing bacteria. Colonization by these bacteria is a source to persistent and resistant Infections, unless removed from the patient (Costerton *et al.,* 1999). Due to the extensive use of these devices in hospitalized patients there is an increased incidence of Device related infections. Management of these infections has now become a huge challenge (Costerton *et al.,* 1999).

Biofilms on indwelling medical devices may be composed of gram positive or gram negative bacteria or yeasts. Bacteria commonly isolated from these devices include the gram positive *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus viridans*; and the gram-negative Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, and Pseudomonas aeruginosa and fungal organisms like Candida species (Roodney, 2001). These organisms may originate from the skin of patients or health- care workers, tap water to which entry ports are exposed, or other sources in the environment. Biofilms may be composed of a single species or multiple species, depending on the device and its duration of use in the patient (Roodney, 2001).

A number of tests are available to detect biofilm formation by microorganisms methods include tissue culture plate method, Congo red agar method, tube method and bioluminescence assay. These methods are often subject to severe analytical limitations and are unable to detect bacterial adherence accurately. In this study, we screened different clinical isolates from medical devices by the three phenotypic methods i.e., TCP, TM, and CRA methods for determining their ability to form biofilms and also evaluated the reliability of these methods in order to determine most suitable screening method.

Materials and Methods

Study centre

The present study was carried out at Department of Microbiology, Gandhi Hospital, Hyderabad.

Study period

The study was conducted over a period of six months from March 2016 to August 2016.

Study type

Prospective, Cross sectional study.

Sample size

100 patients either of sex; neonates, infants& children admitted to the intensive care units irrespective of Immune status.

Inclusion criteria

Patients admitted to the Intensive care units at Gandhi Hospital for women and children.

Patients with medical devices inserted for more than twelve hours.

Exclusion criteria

Patients without medical devices.

Patients in whom medical device was inserted outside the hospital.

Medical device removed with less than twelve hours of duration.

Specimens collected

Intravenous catheters tips (IVC)
Endotracheal tubes (ETT)
Inter costal drain tubes (ICD)
Nasogastric tubes (NGT)

Identification of organism

The organisms isolated were identified by standard biochemical reactions.

Detection of Biofilm Formation (Mathur *et al.,*)

Congo Red Agar method, (CRA)

This a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium, described by Freeman *et al.,* CRA medium is prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar No. 1 10 g/L and Congo Red indicator 8 g/L. First Congo red stain is prepared as a concentrated aqueous solution and autoclaved (121ºC for 15 minutes) separately from the other medium constituents.

Then it was added to the autoclaved brain heart infusion agar with sucrose at 55ºC.5 CRA plates were inoculated with test organisms and incubated at 37ºC for 24 h aerobically.

Black colonies with a dry crystalline consistency indicated biofilm production. The experiment was performed in triplicate and repeated three times.

Tube method

This is a qualitative method for biofilm detection, described by Christensen *et al.,* (1985) A loopful of test organisms was inoculated in 10 mL of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong. The experiment was performed in triplicate and repeated three times.

Tissue culture plate method

This quantitative test is considered the gold-standard method for biofilm detection, described by Christensen *et al.,* (1982) Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth with 1% glucose. Broths were incubated at 37ºC for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well-flat bottom polystyrene tissue culture treated plates were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37ºC for 24 h. After incubation, contents of each well were

removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA auto reader (model 680, Biorad, UK) at wavelength 570 nm. The experiment was performed in triplicate and repeated three times.

Calculation for optical density for detection of Biofilm

Strong biofilm producer = (4× ODc) < OD_{570}

Moderate biofilm producer = (2× ODc) < $OD_{570} \leq$ (4× ODc)

Weak biofilm producer=ODc< $OD_{570} \leq$ (2× ODc)

No biofilm producer = $OD_{570} \leq$ ODc

{OD cutoff (ODc) = average OD_{570} of negative control + (3× standard deviation of negative control)}

Positive Control Biofilm Producer – *Pseudomonas aeruginosa* A.T.C.C. strain 27853.

Negative Control Non Biofilm producer- *Staphylococcus aureus* A.T.C.C strain 25923.

Results and Discussion

The study included 100 patients from Intensive care unit with devices inserted for more than 12 hours. Out of the 100 specimens processed 57 were culture positive, and the rest 43 did not yield any growth in culture.

The specimens included 36 Intravenous Catheter tips, 46 endotracheal tubes, 08 intercostal drain tubes, 10 Nasogastric tubes.

Out of the 57, positive cultures, 52 isolates were bacteria and 5 *Candida* sp. Only the bacterial isolates were subjected for detection of biofilm production. 42 bacterial isolates (80.7%) were found to be biofilm producers.

From the I.V.C's, 16 (80%), of isolates were biofilm producers, from E.T.T's 24(86%) of isolates were biofilm producers, and from N.G.T.'s 1(25%) and I.C.D. tubes 1 (20%) were biofilm producers.

In the present study, the frequency of biofilm production among the different devices, maximum numbers of biofilm producers were isolated from the endotracheal tubes (86%).

In the present findings, the microbial profile of the intra venous catheter, maximum no. of biofilm producers isolated from the I.V.C's were the *S. epidermidis* (45%) among different microorganisms. Whereas in ETT most common organism producing biofilm was *Klebsiella pneumoniae* (36%).

Out of the 4 isolates from the N.G.T. 1 isolate of Klebsiella pneumonia was a biofilm producer. Out of the 5 isolates from the I.C.D's 1 isolate of *Acinetobacter baumanii* was a biofilm producer.

Out of the 52 isolates subjected for Biofilm Production, T.C.P. Method detected 42 (80.7%), T.M. Method detected 38 (66%), C.R.A. Method detected 8 (14%) of the Biofilm Producers.

CRA method showed very little correlation with corresponding methods and the parameters of sensitivity, whereas TCP method shows high sensitivity, specificity and accuracy than the other two methods (Refer Table 1–11 and Fig. 1–10).

Table.1 Biofilm production of isolates

S. No	Organism	Number	Percentage
1	Positive Culture	52	100%
	(Bacteria)		
2	Total No of Biofilm	42	80.7%
	Producers		

Table.2 Specimen wise biofilm production by isolates

S. No	Specimen	Positive Culture	Biofilm Production	Percentage
1	ETT	28	24	86%
2	IVC	20	16	80%
3	ICD	5	1	20%
4	NGT	4	1	25%

Table.3 Frequency between biofilm producers and non biofilm producers

S. No	Specimen	Biofilm Producers	Percentage	Non biofilm Producers	Total
1	ETT	24	86%	4	28
2	IVC	16	80%	4	20
3	NGT	1	33%	3	4
4	ICD	1	25%	4	5
5	Total	42	--	15	57

Table.4 Microbial profile of biofilm producers from IVC

S. No	Organism	Biofilm Producer	Non Biofilm Producer	Percentage
1	Staphylococcus epidermidis	9	0	45%
2	Pseudomonas aeruginosa	3	0	15%
3	Staphylococcus aureus	2	1	10%
4	Klebsiella pneumoniae	1	0	5%
5	Escherichia coli	1	0	5%
6	Proteus mirabilis	0	1	0

Table.5 Microbial profile of biofilm producers from ETT

S. No	Organism	Biofilm Producer	Non Biofilm Producer	Percentage
1	*Klebsiella pneumoniae*	10	1	36%
2	*Acinetobacter baumanii*	6	0	21%
3	*Pseudomonas aeruginosa*	4	0	14%
4	*Escherichia coli*	2	1	7%
5	*Staphylococcus epidermidis*	1	0	3%
6	*Staphylococcus aureus*	1	0	3%

Table.6 Microbial profile of biofilm producers from NGT

S. No	Organism	Biofilm Producer	Non Biofilm Producer	Percentage
1	*Klebsiella pneumoniae*	1	0	25%
2	*Escherichia coli*	0	1	0%

Table.7 Microbial profile of biofilm producers from ICD

S. No	Organism	Biofilm Producer	Non Biofilm Producer	Percentage
1	*Staphylococcus epidermidis*	0	2	0%
2	*Klebsiella pneumoniae*	0	1	0%
3	*Acenitobacter baumanii*	1	1	50%

Table.8 Methods for detection of biofilm production (n=52)

S. No	Method	Number	Percentage
1	CRA Method	8	14%
2	Tube Method	38	66%
3	TCP Method	42	80.7%

Table.9 Comparison between three methods for detection of biofilm production

Method	X^2	p value	Significance
CRA & TCP Method	44.5	< 0.0001	Significant
CRA & TM Method	35	< 0.0001	Significant
TM & TCP Method	0.8	>0.5	Not Significant

Table.10 Grades of biofilm production by TCP Method

S. No	Bio Film Formation	Number	Percentage
1	Strong	10	23%
2	Moderate	26	62%
3	Weak	6	14%

Table.11 Grades of biofilm production by tube method

S. No	Bio Film Formation	Number	Percentage
1	Strong	4	10.5%
2	Moderate	28	66%
3	Weak	8	19%

Fig.1 Maki's roll plate method – *Staphylococcus aureus* (GPC)

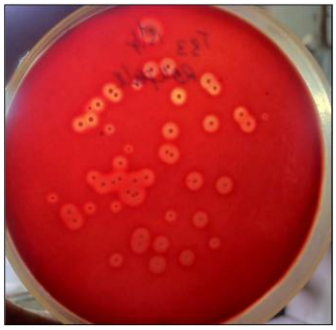


Fig.2 Maki's roll plate method – *Klebsiella pneumoniae* (GNB)

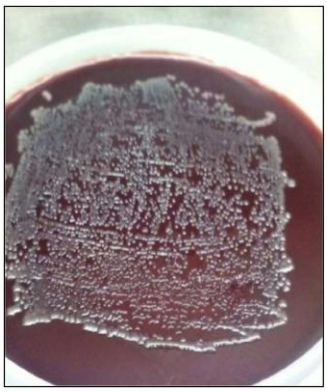


Fig.3 Qualitative culture method

Fig.4 Antibiogram of Gram positive bacteria on Mueller Hinton Agar

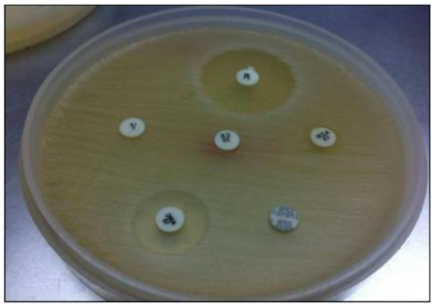


Fig.5 E -Test – Vancomycin

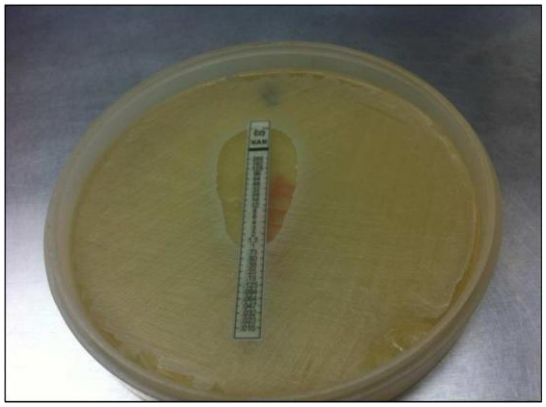


Fig.6 Antibiogram of Gram negative bacteria on Mueller Hinton Agar

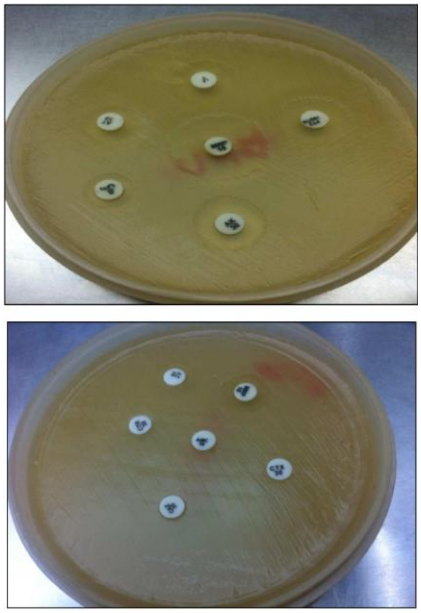


Fig.7 Congo red agar-black coloured colonies biofilm producer

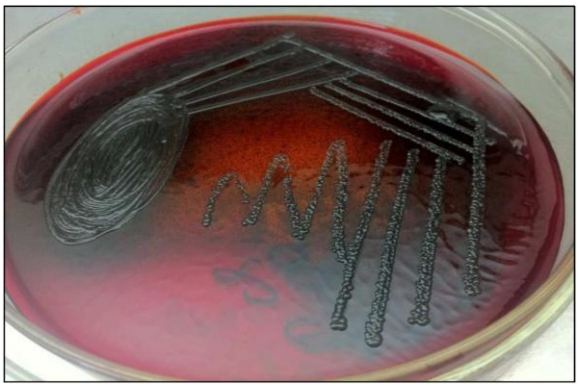
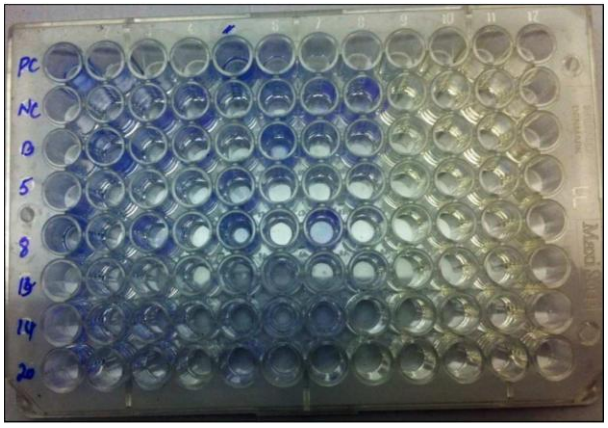


Fig.8 Red/orange coloured colonies non biofilm producer

Fig.9 Tube method

Fig.10 Tissue culture plate method



Out of the 42 isolates which produced Biofilms by T.C.P. Method, 10 (23%) were strong, 26 (62%) were moderate & 6 (14%) were weak biofilm producers. Out of the 38 isolates which produced biofilms by T.M., 4(10.5%) were strong, 28 (66%) were moderate, 8 (19%) were weak biofilm Producers.

Among microorganisms 42% of P. aeruginosa isolates, 37.5% of *A. baumanii*, 25% *E. coli*, 14% of *K. pneumoniae*, 7.60% of *S. epidermidis* were among the strongest biofilm producers.

In the present study, out of the 52 bacterial isolates, 42, i.e., 80.7% were biofilm producers and the highest number of biofilm producers were from the E.T.T.'s (86%), followed by I.V.C.'s (80%), N.G. tubes (25%) and I.C.D. tubes (20%).

Klebsiella pneumoniae was the commonest organism isolated from E.T.T.'s, out of the 11 isolated 10 were biofilm producers. *S. epidermidis* was the commonest organism isolated from I.V.C's and out of the 9 isolated all the 9 were biofilm producers. Similarly out of the 4 isolates from N.G.T'S 1 *Klebsiella pneumoniae* formed biofilm. Out of the 6 isolates from the I.C.D. tubes 1 *Acinetobacter baumanii* isolate, showed biofilm production. *Pseudomonas aeruginosa* and *Acinetobacter baumanii* were among strong biofilm producers.

In the present study detection of biofilm production was carried out by three phenotypic methods. Congo Red Agar method (C.R.A.), Tube method (T.M.) and Tissue Culture Plate, (T.C.P.). The grading of the biofilms was done according to Mathur *et al.,* (2006). Tissue culture plate method was a quantitative and the best method among the other two methods for the detection of biofilm production and detected 42, (80.7%) biofilm producers. Tube Method detected 38, (66.6%) and Congo Red Agar method detected least number of biofilm producers i.e., 8 isolates. (14%). This pattern of biofilm detection is similar to the one demonstrated by Turkyilmaz S *et al.,* (2006) who detected biofilm formation in 61.1% samples by Congo Red agar method, 55.5% by Tube method and 50.5% by Tissue culture plate method in isolates of *Staphylococcus aureus*.

Out of the 42 biofilm producers detected by T.C.P. method, 10(23%), 26(62%), 6(14%), were strong, moderate, weak biofilm producers respectively. Tube method detected 6(14%), 28(66%), 8 (19%) as strong, moderate and weak biofilm producers respectively. Congo Red Agar method detected only 8 (14%) biofilm producers, degree of the biofilm producers could not be appreciated with this method.

C.R.A. method is an easy screening method for biofilm detection but categorization between weak or non-biofilm production was a problem. Similar problem was also experienced with Tube method. An advantage with the T.C.P. method is that it obviates the difficulty of categorization by taking the measurement of the Optical Density (O.D.) value which helps in quantification of the biofilm which is produced. It was recommended by Mathur *et al.,* (2006) that T.C.P. method as an accurate and reproducible method for detection of biofilm producers. In their study T.C.P. method detected 53.9% of biofilm producers, Tube method correlated well with T.C.P. method for strong biofilm producers but it showed difficulty in discriminating between weak and non-biofilm producers. C.R.A. method detected only 1.97% of biofilm producers and this method was not at all recommended for detection of biofilm production.

In the present study p value was <0.0001 between C.R.A. and T.C.P. method and p value was <0.0001 between C.R.A. and Tube method which was significant. p value between Tube method and T.C.P. method was >0.5 showing non significance between both tests.

Hence T.C.P. method was the most sensitive and accurate method among the other two methods studied.

According to a study done by Adilson Oliveira *et al.,* (2010) T.C.P. method detected 81% of biofilm producers, Tube method detected 82% & C.R.A. method detected 73% of the biofilm producers.
In a study conducted by Afreenish Hassan *et al.,* (2011), T.C.P. method detected 64%, Tube method detected 49% and C.R.A. method detected only 3.6% of the biofilm producers.

Samanth Sharvari *et al.,* (2012), T.C.P. method detected 43.2%, Tube method detected 36.2%, and C.R.A. method detected 25.3% of the biofilm producers. According to Nebajit Deka (2014), T.C.P. method detected 83% of biofilm producers, Tube method detected 57% of biofilm producers & C.R.A. method detected 20%.of the biofilm producers. Kanabolouche *et al.,* (2002), detected only 3.8% biofilm producers with C.R.A. method, hence they did not recommend the C.R.A. method for biofilm detection.

Our data indicates that the TCP method is an accurate and reproducible method for screening and this technique can serve as a reliable quantitative tool for determining biofilm formation by clinical isolates of different devices.

To conclude, biofilms are a major cause of recurrent and recalcitrant bacterial infection, leading to increased morbidity in the patient, increased duration of hospital stay and increased economic burden and drain on resources. Of the three phenotypic methods used to detect biofilm formation, Tissue culture plate method (TCPM) is a method with good reproducibility and good specificity. This method can be used routinely in the microbiology laboratory to detect biofilm formation, while our study did not recommend C.R.A. method for detection of biofilms, due to its poor specificity.

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Conflict of Interest

The authors have no conflict of interest

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