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Comparative Study of Different Methods for Biofilm Formation Capacity of Bacteria Causing Respiratory Tract Infection

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Abstract:

Background & Objective: Bacteria tend to live in a community-like assembly called biofilm on poly matrix surfaces. Microorganisms growing in a biofilm are associated with chronic and recurrent human infections and are highly resistant to antimicrobial agents. There are various methods to detect biofilm production like Tube method (TM), Congo Red Agar method (CRA), Micro Titer Plate Method (MTP), bio luminescent assay, piezoelectric sensors, and fluorescent microscopic examination. Objective of This study was conducted to compare three methods for the detection of biofilms.

Method: The study was carried out from May 2023 to October 2023 for checking biofilm formation capacity of respiratory tract pathogens. A total of 300 clinical isolates were subjected to biofilm detection methods. Isolates were identified biochemically by standard microbiological procedures. Antibiotic susceptibility test of isolates was performed by using the Kirby-Bauer disc diffusion technique according to CLSI guidelines. Biofilm detection was tested by TM, CRA plate method, MTP method.

Results: The MTP method was considered to be superior to TM and CRA plate method. From the total of 300 MDR clinical isolates, MTP method detected 83% as high, 12.33% moderate and 4.66% as weak or non-biofilm producers. As it gives good results as compare to the TM and CRA plate method. it was also observed that higher antibiotic resistance shown in biofilm producing bacteria than non-biofilm producers.

Conclusion: We can conclude from our study that the TCP method is a more quantitative and reliable method for the detection of biofilm forming microorganisms as compared to TM and CRA methods, and it can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

I. INTRODUCTION

Bacteria, which live in a community-like assembly called biofilm. Development of bacterial biofilms occurs in a dynamic process that includes i) bacterial attachment to a particular surface, ii) irreversible binding and formation of a hydrated matrix of polysaccharides and protein, iii) formation of slimy layer of biofilm (Aparna MS *et al*; 2008, Panda PS *et al*; 2016). Surfaces that favor biofilm development include inert surfaces as medical devices like, tracheal pipe, Endo-tracheal tip (ET tip), catheter and dead tissues as dead bone fragments (Ward *et al*; 1992). Antibodies are generated in response to the antigens released by the bacteria located in the biofilm. However, these antibodies are unable to kill the bacteria embedded within the biofilm even people with high immune responses (Cochrane DMG *et al*; 1998).

Biofilm formation is the major virulence factors associated with organisms which were adherence and colonize on artificial materials.7 Biofilm production aids bacterial virulence through numerous pathogenic mechanisms as it facilitates attachment to solid surfaces, evasion of phagocytosis and gene exchange between the biofilm´s members generating more virulent strains. Moreover, biofilms can protect bacteria from antimicrobial agents resulting in resistant infections that carry a great clinical significance (Ruchi T *et al*; 2015). The biofilm protects against the action of antibiotics administered for the treatment to cure infections (Otto M, 2008). Biofilm-associated bacteria are less susceptible to antibiotics than planktonic bacteria. This can be explained by different mechanisms like, reduced penetration of the antibiotic inability to reach the bacteria present at the deep part of the biofilm, the weak binding of antibiotics or antibodies to biofilm components, high bacterial density within biofilm, and altered gene expression in the bacteria present in the biofilm (Pinheiro L *et al;* 2014, Stewart PS, 2001).

Antibiotic resistance in respiratory tract infection (RTI) is either community or healthcare acquired, threatening clinical problem faced by treating physicians specially for patients admitted in ICU and NICU (Abdallah NM *et al*; 2011). Biofilms are commonly associated with indwelling devices such as catheters, ET tip, food pipe leading to resistant RTI. Furthermore, biofilms may attach to respiratory tract and its anatomical structures resulting in chronic and recurrent RTI with increased morbidity and economic burden (Ruchi T *et al*; 2015). Therefore, detection of biofilm production by respiratory pathogens can assist the physicians to initiate the proper antimicrobial treatment for patients with RTI. Various laboratory methods for detection of biofilms were

developed for biofilm associated infections. Phenotypic detection of biofilm production can be conducted by various techniques as, Tube Method (TM), Congo Red Agar (CRA) plate method and Micro Titer Plate method (MTP) (Hassan A et al; 2011). biofilm formation by pathogens usually focused on catheterized patients investigated by previous study (Trautner BW *et al*; 2004, Niveditha S *et al*; 2012). This study was performed trying to detect biofilm producing pathogens isolated from clinical samples of respiratory tract (sputum, BAL fluid, pleural fluid, tracheal aspirate) and to evaluate three in vitro Phenotypic methods (TM, CRA and MTP) that can be applied in laboratory for biofilm detection.

II. RESEARCH METHODOLOGY

A) Sample Collection

A prospective study was performed at the Department of Microbiology, Surat Municipal Institute of Medical Education and Research (SMIMER), Surat, Gujarat from August 2022 to May 2023. During the study period, Clinical samples were collected from patients showing clinical manifestations of RTI and transported to the laboratory for immediately process.

B) Microbiological Processing

Received samples including Sputum, BAL fluid, Pleural fluid, Tracheal aspirate, ET tips were initially examined by standard microbiological techniques. Pleural fluid, and ET tips were incubated in BHI broth at 37°C for 24 hours to get sufficient colonizing bacteria. samples were inoculated on Nutrient agar plates, Blood Agar plates and Mac conkey's agar plate that were then incubated at 37°C for 24-48 hours. The isolates were identified by colonial morphology, Gram staining and biochemical reactions by standard microbiological procedure.

C) Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by Kirby Bauer disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guidelines against these antimicrobials: Azithromycin(15-mcg), Erythromycin(15-mcg), Roxithromycin(30mcg), Clarithromycin(15-mcg), Penicillin(10-unit), Ampicillin(30-mcg), Methicillin(5-mcg), Cefuroxime(30-mcg), Cefotaxime(30-mcg), Amikacin(30-mcg), Chloramphenicol(30-mcg), Clindamycin(2-mcg), Linezolid(30-mcg), Ciprofloxacin(5-mcg), Levofloxacin(5-mcg), Ertapenem(10mcg), Meropenem(10-mcg), Co-trimezole(25-mcg), Vancomycin(30-mcg), Rifampicin(5-mcg). The antimicrobial discs were selected on the basis of CLSI guidelines 2022. Isolate which gives resistances towards more than two antibiotic group were selected as MDR and tested for their biofilm production. 300 isolates were selected for their biofilm production (CLSI 2022).

D) Detection of Biofilm Production

Biofilm production by isolated pathogens was detected by three methods which included TM, CRA plate method and MTP method. Reference strains of *Staphylococcus epidermidis* ATCC 12228 and *Staphylococcus epidermidis* ATCC 31484 were also included as negative and positive control strains respectively. Biofilm production was graded into strong, moderate and non/weak. Strong and moderate results were interpreted as positive biofilm production, while, non/weak results were interpreted as negative biofilm production.

I) Tube Method

In tube method, A loopful of the isolated bacteria from overnight cultured media was inoculated in each glass test tube containing 10 ml of trypticase soy broth with 1% glucose. The inoculated test tubes were then incubated at 37°C for 24h. After incubation for 24 hours, tubes were drained and washed with 1% phosphate buffer and allow to dry. 0.1% Crystal violet was used to stain the dried tubes for 15 minutes. Excess stain was then removed by washing the tubes with deionized water and dried in inverted position. Presence of a visible blue film lining the bottom and the wall of the tube indicated positive result for biofilm production. Formation of a stained ring only at the interface was consider as negative result (Christensen GD et al; 1982, Bose S et al; 2009).

II) Congo Red Agar Method

Congo red agar is a medium composed of brain heart infusion (BHI) broth (37 g/l) supplemented with sucrose (50 g/l), Agar (10 g/l) and Congo red (0.8 g/l). Cconcentrated aqueous solution of the Congo red stain prepared a that was separately autoclaved at 121ºC for 15 minutes. Then it was added to the autoclaved BHI agar with sucrose at 55ºC. Prepared CRA plates were inoculated with the isolated pathogens and incubated at 37°C for 24-36 hours. (Hassan A et al; 2011, Freeman J et al; 1989) Growth of black dry crystalline colonies on the CRA plates indicated biofilm production while a pink or red colored colonies indicates biofilm non-producer (Ruchi T et al; 2015).

III) Micro Titre Plate Method (MTP)

MTP is the gold standard test for detection of biofilm formation (Hassan A et al; 2011). A loopful of freshly cultured single colony of isolate was inoculated in 10 ml of trypticase soy broth with 1% glucose. The inoculated broth was kept in the incubator at 37°C for 24 hours. After 24 hours, Bacterial suspensions were further diluted 1:100 with fresh medium. Separate wells of a sterile microtiter tissue culture plate, composed of 96 flat bottom wells, were filled with 200 µl of the prepared diluted bacterial suspension. Similarly, control organisms were put in the individual well. Sterile broth was used for sterility and to identify non-specific binding. After incubation, the plate was gently tapped to remove the content of the wells and washed with 200 µl of phosphate buffer. The washing step was repeated three to four times to remove any free bacteria present in the wells.
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. (Turkyilmaz S *et al*; 2006) Optical density (OD) of stained adherent biofilm was obtained by using micro-ELISA auto reader at wavelength 570 nm. The interpretation of biofilm

production was done according to the criteria of Christensen GD. Test was carried out in triplicate and average of three OD values was taken. Optical densities values indicated bacterial adherence to the wells and biofilm formation. The OD values were calculated and biofilm production was graded into strong, moderate and non/weak (Table 1) as described in previous studies (Panda PS, et al; 2016, Christensen GD *et al*; 1985).

[Table 1: Classification of bacterial biofilm adherence by MTP method]

Optical density	Adherence	Biofilm formation
<0.120	None	None/Weak
0.120-0.240	Moderate	Moderate
>0.240	Strong	High

III. Statistical Analysis

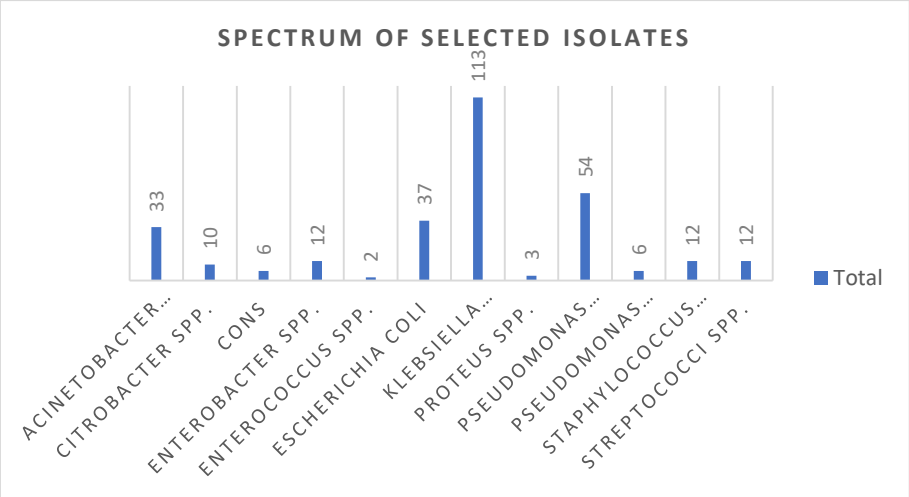
The statistical analysis was performed using SPSS software version 25.0. Data were presented as percentages and proportions. The Chi-square test was applied when two or more set of variables were compared. The critical value of *P*-value indicating the probability of significant difference was taken as <0.05.

MTP method was considered as the gold standard method for biofilm detection based on the available review literature. Accordingly, the data of MTP method were compared with TM and CRA. The data were presented as numbers and percentages. Parameters like sensitivity, specificity and positive predictive value (PPV) and negative predictive value (NPV) were calculated for each test by using Greenhalgh´s formulas (Greenhalgh T *et al*; 1997).

IV. RESULTS AND DISCUSSION

A total of 300 MDR isolates from patients with suspected RTI were processed for their Biofilm Formation in the study. *Escherichia coli* was the commonest isolate encountered in our study is *Klebsiella pneumoniae* (37.66%) followed by *Pseudomonas aeruginosa* (18%) was the predominant.

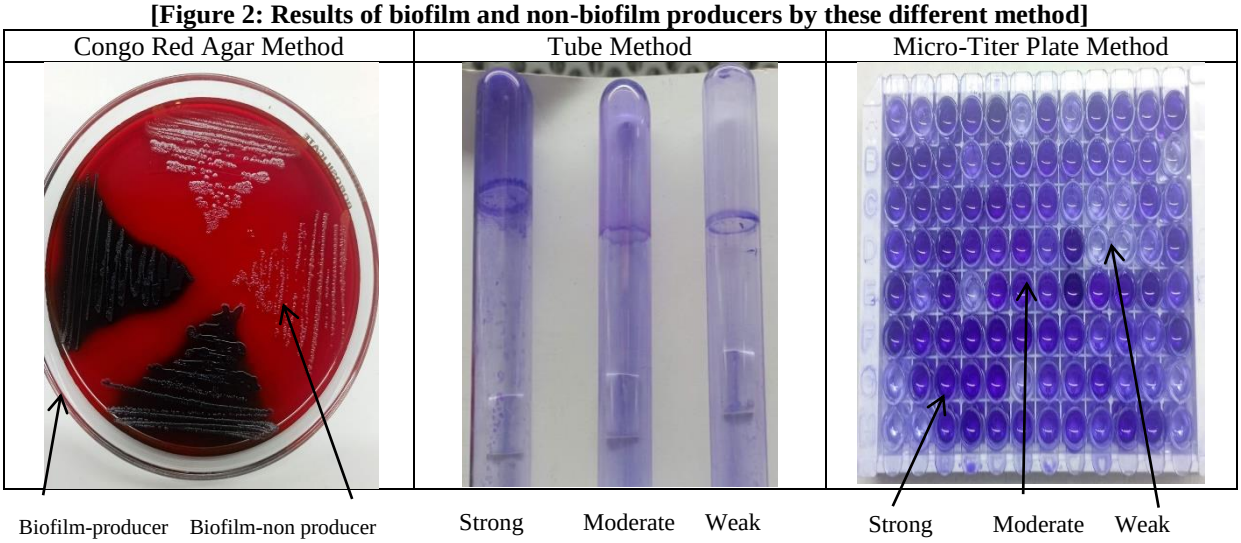
[Figure: 1 No. of isolates used for the study]



Among 300 isolates, MTP method is a standard method, detected 249 as strong and 37 as moderate biofilm producers. The majority of the pathognic organisms associated with biofilm production were *K. pneumoniae* (36.66%), followed by *P. aeruginosa* (17.66%), *E. coli* (11%), and *Acinetobacter spp*. (10%).

Strong biofilm production was caused mainly in immune-compromised patients, sensitive predominantly to meropenem, vancomycin and linezolid. By TM, the number of strong biofilm producers were 89, moderate was 111, 50 were weak and 50 were non-biofilm producers. Very different results were observed by the CRA method, in which only 87 isolates showed black colonies with crystalline appearance which is indication of bio-film producers as compare with red colonies of biofilm- non producers.

[Figure 2: Results of biofilm and non-biofilm producers by these different method]



[Table 2: Comparative Grading of biofilm by Different Three Methods]

Biofilm formation	Micro-Titer plate Method	Tube Method	Congo Red Agar Method
Strong	249 (83%)	89 (29.66%)	87 (29%)
Moderate	37 (12.33%)	111 (37%)	0 (0%)
None/Weak	14 (4.66%)	100 (33.33%)	213 (71%)
Total	300	300	300

[Table 3: Performance Characteristics of Tube Method and Congo Red Agar Plate Method as compare with Micro Titer Plate Method]

Method	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Accuracy
Tube Method	76.88%	100%	100%	14%	77%
Congo Red Agar Plate	58.96%	100%	100%	6%	60%

DISCUSSION

Antimicrobial resistant bacteria (AMR) lead to be a major challenge for all in clinical treatment. The production of biofilm is considered as one of the major causes of antimicrobial resistance in pathogens. Bacteria, which are embedded in the biofilm can survive at higher concentration of antibiotics (Sultan *et al.;* 2019). In the present study, we processed for samples of respiratory tract infections and investigated by standard methods to check the ability of isolates to form biofilm by three in vitro Phenotypic method on laboratory basis. A total 300 MDR bacterial isolates were used in our study. Gram-negative bacteria found to be the predominant constituting 89% of the total isolates. *Klebsiella pneumoniae* (37.66%), followed by *Pseudomonas aeruginosa* (18%), Escherichia *coli (*12.33%) and *Acinetobacter spp.* (11%) *was* the most prevalent organism isolated from the samples (Sputum, BAL Fluid, Pleural Fluid, Tracheal Aspirate). All 300 MDR isolates were further subjected to TM, CRA, MTP methods for Phenotypic detection of biofilm production. The MTP method is the gold standard method, detected high biofilm formation in 249 out of 300 bacterial isolates (83%). We chose these in vitro methods because they can be performed in most laboratory settings. The TM detected strong biofilm production in 89 isolates (29.66 %) while the CRA detected biofilm production in 87 isolates (29%).

Biofilm production studied on total 110 isolates by Hassan *et al*., in 2011, reported that the CRA method detected 11 isolates, TM in 54, and 70 isolates in MTP method were biofilm producers. Baqai *et al*., studied that 75% isolates produce biofilm by Tube Method and 10% isolates were detected as biofilm producers in CRA plate method. Mathur *et al*., use 152 isolates tested for their ability for biofilm formation, out of them, 47.3%, 41.4% and 5.2% isolates were biofilm producers as detected by MTP, TM and CRA respectively. Knobloch *et al*., studied that out of 128 isolates of *S. aureus*, CRA detected only 3.8% of isolates were biofilm producer as compared to MTP is 57.1% as biofilm producers so, they were not recommended the CRA method is preferable for biofilm detection.

[Table 4: Relevant results of biofilm production test]

Sr. no	Positive Biofilm production by			Autor/ publication year
	TM	CRA	MTP	
1.	49%	11%	63%	Hassan *et al.*, 2011
2.	41.4%	5.2%	47.3%	Mathur *et al.*, 2006
3.	53.7%	43.5%	-	Ruzicka *et al.*,2004
4.	-	3.8%	57.1%	Knobloch *et al.*, 2002

We have performed the MTP method by adding of 1% glucose as source of sugar in trypticase soy broth. Addition of glucose helps to enhance biofilm formation. It was also reported in study conducted by Mathur et al., in 2006 and Bose *et al*., in 2011. In this study TM is 76.88 % sensitive, 100 % specific and 77% accurate for biofilm detection. This method correlated well with MTP for identifying strong biofilm producers. Tube Method cannot be suggested as screening test for identify biofilm producing isolates for biofilm production according to the all-other previous study. Biofilm production checked by Ruzicka *et al.,* by two different methods. In this out of 147 isolates of *S. epidermidis*, TM detected biofilm formation in 79 (53.7%) and CRA detected in 64 (43.5%) isolates which showed that TM is better for biofilm detection than CRA. Baqai et al. tested TM to detect biofilm formation. According to their results, 75% of the isolates not able for biofilm formation with the CRA method. The CRA method showed very little correlation with the other methods and parameters of sensitivity (58.96%), specificity (100%) and accuracy (60%) were low. So, Knobloch *et al.,* did not recommend the CRA method for biofilm detection in their study to check biofilm production. The main reason is that, out of 128 isolates *S. aureus*, CRA detected only 3.8% positive for biofilm formation as compared to MTP method in which 57.1% detected as biofilm producing bacteria.

V. CONCLUSION

It is found in his study, that TM and CRA correlated well with MTP method for strong biofilm detection but not suitable method for moderate and non/weak biofilm detection. This could be accredited to the subjective assessment used in TM and CRA in comparison to the objective grading scheme used in MTP method. The CRA have lower sensitivity and specificity results than those of TM. The MTP to be the gold standard and most specific Phenotypic test for detection of biofilm production in the present study. It was also an easy test to perform at the laboratory basis and it detected the biofilm production by both qualitative and quantitative manners. The interpretation of the MTP method conducted by ELISA reader, which eliminates the subjective errors seen with other Phenotypic tests and gives accurate results.

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