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Original Article

Microbiology Section

# Comparative Study of Biofilm Formation in *Pseudomonas aeruginosa* Isolates from Patients of Lower Respiratory Tract Infection

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## ABSTRACT

**Background:** This study assessed biofilm formations of *P.aeruginosa* which was isolated from patients with Lower Respiratory Tract Infections (LRTIs).

**Objective:** This study was conducted to compare different methods of biofilm formations seen in *P. aeruginosa* which was obtained from LRTI patients.

**Materials and Methods:** In this cross-sectional study, we investigated a total of 80 *P. aeruginosa* isolates obtained from LRTI patients by different methods. Tube method (TM), tissue culture plate (TCP) method and modified tissue culture plate (MTCP) method. They were subjected to biofilm detection methods.

**Results:** The MTCP method produced a higher accuracy ratio than TCP method. In terms of sensitivity and specificity, the MTCP method was considered to be superior to TM. We observed a higher antibiotic resistance in biofilm producing bacteria than in non-biofilm producers.

**Conclusion:** In our study, MTCP was found to be more sensitive and specific method for biofilm detection than TCP and TM.


**Keywords:** Biofilm formation, *P. aeruginosa*, Tissue culture plate, Lower respiratory tract infection

## INTRODUCTION

*Pseudomonas aeruginosa* is an important pathogen which causes life threatening infections in patients who suffer from respiratory diseases. These infections are hard to treat, partly due to the high intrinsic resistance of the bacterium to clinically used antibiotics and partly due to the formation of antibiotic tolerant biofilms. Biofilms are known to express genes which are different from those of planktonic cells, and they are generally believed to closely resemble planktonic cells which are in stationary phase [1].

The pathogenesis of *Pseudomonas aeruginosa* in chronic lung infections leads to a decline in lung function and respiratory failure in biofilm formation [2,3]. Biofilm formation may be determined in several ways, but most frequently, it is demonstrated by the tube test [4,5] in which the bacterial film which lines a culture tube is stained with a cationic dye and is visually scaled, or by the microtitre-plate test [5], in which the Optical Density (OD) of the stained bacterial film is determined spectrophotometrically. We screened development of mature biofilm layers formed by *P. aeruginosa* isolates obtained from LRTI patients by three different methods, which could be used in a routine clinical laboratory, for determining their abilities to form biofilms.

## MATERIALS AND METHODS

### Study design and subjects

This cross-sectional study was conducted in the Department of Pulmonary Medicine and Microbiology Department of a tertiary care hospital, Lucknow, between January 2011 and May 2012. Two hundred and fifty patients from indoor and outdoor patient departments of pulmonary medicine, with confirmed diagnoses of lower respiratory tract infections and who did not receive either of the antibiotics in previous 72 hrs, patients of ages ≥ 18 years with symptoms which were suggestive of LRTI, following fever (>100F), cough, production of sputum, leukocytosis (>12000 wbc/mm$^3$ or >15% bands) who gave written informed consents for their participation and their sputum samples for examination, were enrolled for the study. The study was approved by the institutional ethics committee.

### Sample collection and processing

Sputum samples were collected in sterile, wide mouthed containers and then transferred to Microbiology Laboratory for further processing. Samples were cultured onto Pseudomonas isolation agar plates (Hi-media). Colonies with an appropriate colonial morphologies were classified presumptively as *P. aeruginosa* and they were further identified by conventional biochemical tests [6]. Antimicrobial susceptibility was done by Kirby Bauer disk diffusion method as per Clinical Laboratory Standard Institute (CLSI) 2010 guidelines [7]. *P. aeruginosa* isolates were stored in 1% nutrient agar stabs at 4°C for doing further analyses.

### Qualitative and Quantitative biofilm formation assays

**1. Tube method (TM)**

The qualitative assay for biofilm formation was noted according to the method which was described by Christensen et al., [5].

**2. (a) Tissue culture plate (TCP) method**

**(b) Modified Tissue culture plate (MTCP) method**

Both these tests were performed as has been described by Dheepa et al., [8]. The reading was performed two times:

- Before addition of glacial acetic acid, as in standard microtitre-plate test and
- After glacial acetic acid was added, as in modified microtitre-plate test.

The wells were then treated with 160 µL of 33% glacial acetic acid for 15 min at room temperature, to solubilize the dried crystal violet which was adherent to any biofilms.

*P. aeruginosa* PAO1 (Biofilm producing) and *P. aeruginosa* ATCC 27853 (non-biofilm-producing) were used as controls.

For the purpose of doing a comparative analysis of test results, the adherence capabilities of the test strains were classified into four categories: non-adherent (0), weakly adherant (+), moderately adherant (++), or strongly (+++) adherent, based upon the ODs of bacterial films. The cut-off optical density (OD) for a tissue culture-plate is defined as three standard deviations above the mean OD of the negative control. Strains were classified [Table/Fig-1].

| OD values of Tissue Culture Plate method | | | OD values of modified Tissue Culture Plate method | | |
|---|---|---|---|---|---|
| Mean OD value | Adherence | Biofilm formation | Mean OD value | Adherence | Biofilm formation |
| <0.062 | Non | Non | <1.33 | Non | Non |
| 0.062-0.124 | Weak | Weak | 1.33-2.66 | Weak | Weak |
| 0.124-0.248 | Moderate | Moderate | 2.66-5.32 | Moderate | Moderate |
| >0.0248 | Strong | Strong | >5.32 | Strong | Strong |

**[Table/Fig-1]:** Classification of bacterial adherence by microtitre plate method

For all *P. aeruginosa* isolates, the strength of biofilm formation was calculated as per the given formula.

| Average OD value | Biofilm production |
|---|---|
| OD ≤ ODc | Non-adherent |
| ODc < OD ≤ 2 x ODc | Weakly adherent |
| 2 x ODc < OD ≤ 4 x | ODc Moderately adherent |
| 4 x ODc < OD | Strongly adherent |

Optical density cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control.

## STATISTICAL ANALYSIS

The results have been presented in means (±sd) and percentages. The Mann-Whitney U-test was used to compare the continuous variables. The sensitivity, specificity, positive and negative predictive and accuracy values of the tests were calculated. A p-value of <0.05 was considered as significant. STATA, version 8.0 was used for all the analysis [Table/Fig-2].

For the data calculation, we did classification of bacterial adherence, based on three standard deviations above the mean OD of the negative control [8].

## RESULTS

Out of 250 sputum samples, 80 were confirmed as *P. aeruginosa* isolates. Biofilm productions assessed by qualitative tube method revealed 16 (20%) strong biofilm producers, 17 (21.25%) moderate producers, and 47 (58.75%) weak or non-biofilm producers [Table/Fig-3].

| Experimental condition | OD570 Mean+ SD | No.(%) biofilm producers | No. (%) Strong biofilm producer isolates | No. (%) Weak/non biofilm Producer isolates | p-value |
|---|---|---|---|---|---|
| With GAA | 1.06±0.71 | 80 | 19 (23.6) | 61 (76.4) | p=0.0001* |
| Without GAA | 0.18±0.17 | 80 | 16 (20) | 64 (80) | |
| p-value | 0.001* | | 0.0001** | | Moderate |

**[Table/Fig-2]:** Spectrophometric assay of bioflim formation among isolates of *P. aeruginosa* under two experimental conditions

| | Biofim foramtion | TM (%) | TCP(%) | MTCP(%) |
|---|---|---|---|---|
| No of isolates (80) | High | 16(20%) | 5(6.25%) | 7 (8.75 %) |
| | Moderate | 17(21.25%) | 11(13.75%) | 12(15%) |
| | Weak | 29(36.25%) | 48(60%) | 30(37.5%) |
| | None | 18(22.5%) | 16(20%) | 31(38.75%) |

**[Table/Fig-3]:** Overall results of the isolates for biofilm in TM and MTCP test

Quantitative biofilm formation assay done on 80 isolates of *P. aeruginosa* showed that all isolates were better biofilm producers (OD was more than 0.0248) in presence of glacial acetic acid than in absence of glacial acetic acid.

The overall percentage of resistance which was observed among all the *P. aeruginosa* isolates, including those for biofilm positive and biofilm negative isolates and that for resistance to 14 antibiotics which were tested, has been given in [Table/Fig-4]. This table shows that the resistance to antibiotics was more for most of the biofilm positive isolates as compared to that for the biofilm negative isolates. Isolates showed maximum resistance to amoxyclav (97%), followed by ampicillin (95%) and levofloxacin (74%).

Mean level of OD570 was significantly (p=0.001) higher among isolates with GAA (1.06±0.71) than in those without GAA (0.18±0.17). The percentage of strong biofilm producer isolates was 23.6% with GAA and it was 20% without GAA. However, the percentage of weak/non biofilm producer isolates was 76.4% with GAA and it was 80% without GAA. The differences were statistically significant (p=0.0001). Sensitivity and specificity of TM were 84.3% and 58.6% respectively, whereas for MTCP, both these parameters had higher values: 88.6% and 63.9% respectively [Table/Fig-5].

| S. No. | Resistance | | | |
|---|---|---|---|---|
| | Antibiotics | Biofilm positive Isolate (n=44) | Biofilm negative Isolates (n=36) | Resistance of all isolates (n=80) |
| 1 | Ampicillin | 90.9 | 100 | 95 |
| 2 | Amikacin | 31.8 | 33.3 | 33 |
| 3 | Amoxiclav | 93.1 | 97.2 | 97 |
| 4 | Aztreonam | 43.1 | 41.6 | 43 |
| 5 | Ciprofloxacin | 25 | 25 | 25 |
| 6 | Ceftazidime | 70.4 | 55.6 | 64 |
| 7 | Cefepime | 36.3 | 36.1 | 37 |
| 8 | Ceftriaxone | 63.6 | 69.4 | 67 |
| 9 | Gentamicin | 47.7 | 44.4 | 47 |
| 10 | Imipenem | 6.81 | 0 | 4 |
| 11 | Meropenem | 47.7 | 38.8 | 44 |
| 12 | Pipercillin/ Tazobactum | 15.9 | 13.8 | 15 |
| 13 | Tobramycin | 29.5 | 33.3 | 32 |
| 14 | Levofloxacin | 79.5 | 66.6 | 74 |

**[Table/Fig-4]:** Antibiotic susceptibility results (percentage) of *P. aeruginosa* isolates

| Screening method | Sensitivity | Specificity | Positive predictive value (%) | Negative predictive value (%) | Accuracy (%) |
|---|---|---|---|---|---|
| TM | 84.3 | 58.6 | 78.2 | 68.0 | 75 |
| MTCP | 88.6 | 63.9 | 75.0 | 82.1 | 77.5 |

**[Table/Fig-5]:** Diagnostic parameters of TM and MTCP method for biofilm detection

## DISCUSSION

Multidrug-resistant *P. aeruginosa* has been reported worldwide and it has now been recognized to cause one of the healthcare-associated infections which are most difficult to control and treat. We evaluated biofilm formation capability of originate from the sputum of the patients of LRTI.

In the TCP method, the number of isolates which showed strong biofilm formations was 5 (6.25%) and weak biofilm producers were 48(60%).

Our findings differed from those of Hassan et al., who reported that the TCP method had shown the biofilm formation to be 70 (64.7%), and non or weak biofilm producers to be 40 (36.3%)[9].In our study, biofilm producers showed increased resistance to levofloxacin

(79.5%), followed by ceftazidime (70.4%) and meropenem (47.7%) and non-biofilm producers showed increased resistance only for ampicillin and ceftriaxone. A recent study showed significantly higher resistance to ceftazidime, cefepime and pipercillin [8].

By modified TCP method, strong biofilm procedures were found to be 7(8.75%), 12(15%) were moderate producers and 30 (37.5%) isolates were weak biofilm producers. Biofilm production, studied particularly by doing a qualitative biofilm assay by tube method, showed only 20% isolates to be strong biofilm producers. Thus, TM cannot be recommended as a general screening test to identify biofilm-producing isolates [5,10]. Looking simply at percentages, we could arrive at conclusion that TM was the most effective method, but after doing extensive statistical analysis, it was found that modified tissue culture plate method with glacial acetic acid had better sensitivity and specificity as compared to TM.

Our data indicated that MTCP method was an accurate and a reproducible method which could be used for biofilm detection and that this technique could serve as a reliable quantitative tool for determining biofilm formation.

## CONCLUSION

We can conclude from given study, that most of the *P. aeruginosa* isolates obtained from sputum samples of LRTI patients in vitro, had capacities to produce biofilms. MTCP method was found to be the most quantitative, reliable and accurate screening method as compared to TM, for studying biofilm formations.

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