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EVALUATION OF BIOFILM FORMING ABILITY OF THE MULTIDRUG RESISTANT *PSEUDOMONAS AERUGINOSA*

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ABSTRACT
Biofilm formations of 25 clinical isolates of Pseudomonas aeruginosa were screened by Tissue Culture Plate (TCP), Tube Method (TM) and Congo Red Agar (CRA) method. The susceptibility tests showed that majority of the organisms were multidrug resistant with 86%. Based on the biofilm-positive phenotype, the strains were classified as high, moderate and weak or no biofilm was detected. Of the 12 isolates screened, 3 isolates were high biofilm producers, moderate and weak production was seen in 3 isolates for each type. However, TM correlated TCP but was difficult to detect biofilm negative isolates. Screening by CRA method was not remarkable. Hence, it was found that TCP method is highly sensitive method for detection of biofilm producing *P. aeruginosa*

INTRODUCTION

Through the years *Pseudomonas aeruginosa,* a gram negative, non- fermentative bacillus, has emerged as a major pathogen among nosocomial infections among immunocompromised patients (Kinoshita *et al*., 1997, Tsakris et *al*., 2009). Pseudomonas infections can involve any part of the body i.e. respiratory tract infections, bacterial keratitis, bones and joints, gastrointestinal infection [GI], hemalogical malignancies, meningitis and brain damage in some patients, chronic otitis, urinary tract infection and paronychial infection etc. *P. aeruginosa* has an intrinsic high resistance against most antibiotics, which significantly contributes to eradication failure. Different mechanisms accounts for this inherent multi-drug resistance against a range of structurally and functionally different antibiotics such as penicillin, gentamycin, third generation cephalosporin's, carbepenem's like imipenem and meropenem, fluroquinolones like ciprofloxacin, norfloxcin and tetracycline etc. (Kohler *et al*., 1999; Mesaros *et al*., 2007; Pumbwe and Piddock, 2000).

Another important factor contributing to *P. aeruginosa* pathogenesis in clinical settings is the biofilm mode of growth involved in chronic as well as in acute infections (Schaber *et al*., 2007). Though not new to the environment microbiologist, persistent infection due to biofilm formation is certainly a new and additional burden to clinicians who treat infections. *P. aeruginosa* infections are usually caused by bacterial association with the surface of either human tissue or indwelling devices such as catheters, used in respiratory and urinary tracts so that the infections may be regarded as biofilm-associated diseases bacterial. Biofilms are formed from individual free-floating [planktonic] cells and are defined as an exopolysaccharide-surrounded bacterial complex on the biotic or abiotic surface (Hoiby *et al*., 2001). Bacterial cells in the biofilm often display a variety of phenotypic differences from those in the planktonic culture. These include some phenotypic changes such as motility, production of extra cellular polysaccharide and increased resistance to antibiotic and host defense system. *P. aeruginosa* also forms biofilm readily, which may the most important reason why the infections cannot be effectively treated and cured.

In this study we screened 12 multi drug resistant *Pseudomonas aeruginosa* strains by Microtitire plate method or tissue culture plate (TCP), tube method (TM), Congo red agar (CRA) methods for determining the biofilm production and also evaluated the reliability of these detection methods.

MATERIALS AND METHODS

Bacterial strains: Twenty-five strains used in this study were isolated from 40 clinical samples. They were identified to species level as per Gilardi (1978). For each study an overnight culture was inoculated in fresh brain heart infusion (BHI) broth and further incubated to ensure exponential growth conditions.

Antimicrobial susceptibility testing: Testing was performed in accordance with the guidelines established by the Clinical and Laboratory Standards Institute, 2009, with the Kirby-Bauer method using nine antibiotic discs including: Gentamycin,

Aztreonam, Colistin, Polymyxin-B, Tobramycin, Ceftazidime, Cefepime, Imipenem, Piperacillin, Ciprofloxacin and Ticarcillin, (Hi-media, Mumbai) on Mueller Hinton agar medium. The plates were incubated at 37ºC for 24 hr to check the zone of inhibition. Bacterial strains that demonstrated resistance to three or more categories of antibiotics were defined as multi drug resistant (MDR). The *P. aeruginosa* ATCC 27853 strain was used as the standard control.

MIC: MIC's were determined on plates of mueller-hinton broth containing serial two-fold dilutions of each antibiotic. Bacterial suspensions of 10^4 colony-forming units (CFU)/mL were inoculated onto the surface of the plates, and results were recorded after overnight incubation at 35ºC in an aerobic atmosphere. The MIC was defined as the lowest antibiotic concentration with no visible growth (Kelmani *et al.,* 2008).

Biofilm formation assay of *P. aeruginosa*

i) Microtiter Plate Assay Method (MTP)

The microtiter plate assay method described by Christensen *et al*., 1985 is most widely used and was considered as standard test for detection of biofilm formation. Isolates from fresh agar plates were inoculated in Luria Bertani (LB) broth and incubated for 18 hr at 37ºC and diluted 1 in 100 with fresh broth. Individual wells of sterile, polystyrene, 96 well-flat bottom microtitre plate's wells were filled with 0.2 mL aliquots of the diluted cultures and only broth served as control to check the sterility and non-specific binding of media.

The micro titer plates were incubated for 18 hr and 24 hr at 37ºC. 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). Biofilms formed by adherent sessile organisms in 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.

ii) Tube Method (TM)

A qualitative assessment of biofilm formation was determined as previously described by Christensen *et al*., 1985. LB broth (10 mL) was inoculated with loopful of microorganisms from overnight culture plates and incubated for 24 hr at 37ºC. The tubes were decanted and washed with phosphate buffer saline (PBS) of pH 7.3 and dried. Dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were then dried in inverted position and observed for biofilm formation.

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate or 3-strong.

iii) Congo red agar method (CRA)

Freeman *et al.,* 1989 had described an alternative method of screening biofilm formation; which requires the use of a specially prepared solid medium-BHI broth supplemented with 5% sucrose and Congo red. The medium was composed of BHI, sucrose, agar no. 1 and congo red stain. Congo red was prepared as concentrated aqueous solution and autoclaved at 121ºC for 15 minutes, separately from other medium constituents and was then added when the agar had cooled to 55ºC. Plates were inoculated and incubated for 24 to 48 hr at 37ºC.

Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centers of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an intermediate result.

RESULTS AND DISCUSSION

Antibiotic sensitivity test

In the present study, 25 isolates were subjected to susceptibility testing as per Kirby-Bauer disc diffusion method against the diverse group of antibiotics as mentioned earlier. The total percentage (%) of resistance against each antibiotic was represented in Table 1.

The Kirby-Bauer method showed that majority of *P. aeruginosa* strains was multidrug resistant to more than five antibiotics and were found to be susceptible to four major antibiotics - Colistin, Polymyxin- B, Ticarcillin and Aztreonam. The susceptibility pattern was found to the non-traditional antibiotics, as the use of the following is uncertain due to their toxicity effects.

The Table 3 shows the incidence of resistance in *P. aeruginosa* to three different groups of antibiotics- penicillin's, aminoglycosides and flouroquinolones. The isolates showed 100% resistance to Cefepime, Ceftazidime, Piperacillin and

Table 1: Incidence of resistance in *P. aeruginosa* to three different groups of antibiotics

Antibiotics	Incidence of resistance	
	No. of Isolates	Percentage (%)
Aminoglycosides		70.00
Tobramycin (Tb)	11	44.00
Gentamycin (G)	24	96.00
β-Lactams		50.00
Ticarcillin (Ti)	5	20.00
Piperacillin(Pc)	25	100.00
Cefepime (Cp)	25	100.00
Ceftazidime(Ca)	25	100.00
Imipenem(I)	5	20.00
Aztreonam (Ao)	10	40.00
Colistin (Cl)	3	12.00
Polymyxin- B (Pb)	3	12.00
Fluoroquinolones		100.00
Ciprofloxacin (Cf)	25	100.00

Table 2: Screening of 12 *P. aeruginosa* isolates for detection of biofilm formation by Micro titer Plate Assay method, Tube Method and Congo Red Agar method

Number of isolates	Biofilm formation	Screening methods		
		MTP	TM	CRA
Clinical isolates (11)	High	5	5	1
	Moderate	3	3	2
	Weak	3	3	8
Standard isolate (1)	High	-	+	-
ATCC- 27853	Moderate	+	-	-
	Weak	-	-	+

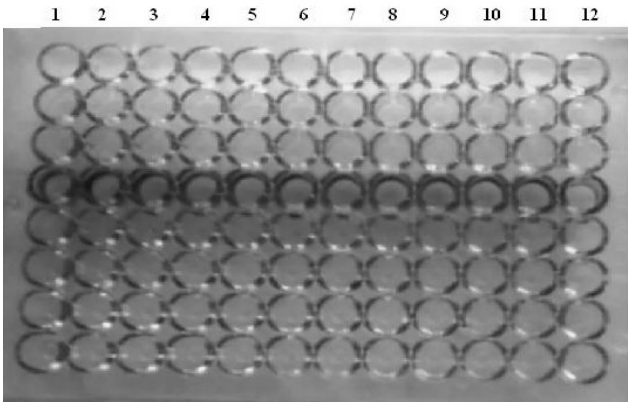

Figure 1: Screening of biofilm producers by Microtiter Plate Assay method; Lanes 1-5, High producers of biofilm; Lanes 6-8, Moderate producers of biofilm; Lanes 9-12, Weak producers of biofilm

Ciprofloxacin. The aminoglycosides-gentamycin and tobramycin showed 96% and 44% of resistance respectively, whereas the non-traditional antibiotics like-Colistin, Polymyxin-B, Ticarcillin and Aztreonam showed resistance in the range of 12%, 12%, 20% and 40% respectively (Table 1).

In the present study the percentage of multi drug resistant strains were significantly high (86%), in comparison to 40-70% reported earlier (Shankar *et al.,* 2009).

The result of this study suggests that the antibiotic use measures are important in combination with traditional infection control measures for preventing the multi-drug resistant pathogens.

Minimal Inhibitory Concentration (MIC) of gentamycin

Among 25 isolates 10 MDR *P. aeruginosa* strains were highly resistant to gentamycin which showed MIC in the range of 16 to >640 μg/mL.

Biofilm formation assay of *P. aeruginosa*

Biofilm production has been reported in strains of all *P. aeruginosa* associated with the infection of biomedical devices (Donlan and Costerton, 2002; Hoiby *et al.,* 2001; Ceri *et al.,* 1999). Investigations to understand the detection and pathogenesis of these infections have been focused upon the adherence of microorganisms to surfaces. In this study we tested 12 clinical isolates of *P. aeruginosa* by three invitro screening procedures for the ability to form biofilm. The microtiter plate assay method, from the total of 12 isolates tested for biofilm formation, high biofilm producers were 5 (41.6%), 4 (33%) were moderate and 3 (25%) isolates were considered as non/weak biofilm producers (Table 2; Fig. 1). The results are in agreement with the observations of other investigators in which only few or no biofilm producing isolates could be detected using this medium.

The tube method showed good correlation with the microtiter plate assay method for strongly biofilm forming isolates and total 6 (50%) isolates were picked up as high and 3(25%) were moderate producers. However, it was difficult to discriminate between moderate and weak biofilm producers 3(25%) (Table 2; Fig. 2). These observations suggested a strong dependence between growth condition and biofilm formation in Pseudomonas.

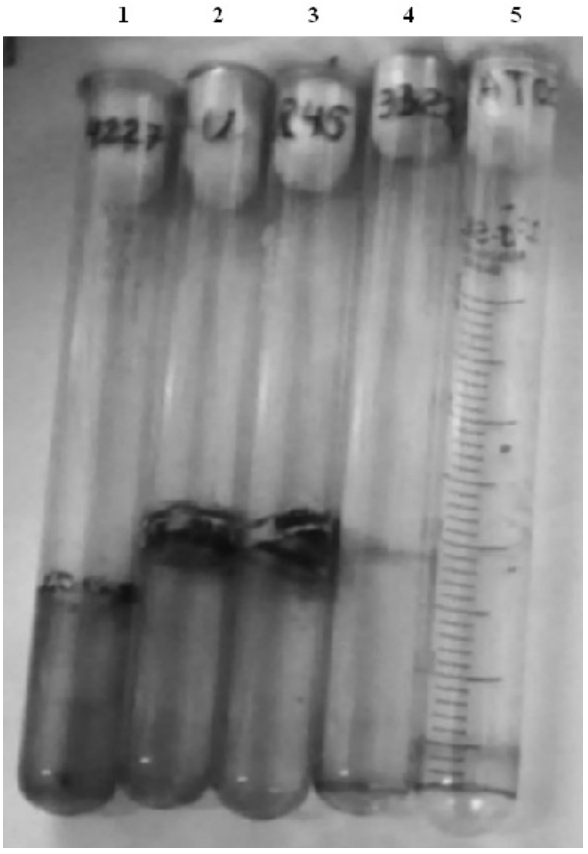

Figure 2: Screening of biofilm producers by Tube Method; Tube 1-2, strain 4227 and U- High producers of biofilm; Tube 3, strain R45-Moderate producer of biofilm; Tube 4-5, strain 3322 and ATCC-Weak producers of biofilm

By Congo red agar method we obtained very different results. Most of the strains displayed pink colonies (weak/non producers) were 9 (75%) in number. 2 (16.6%) were moderate producers showing darkening of colonies with absence of dry crystalline colonial morphology. 1 (8.3%) was high producer with black colony with a dry crystalline consistency (Table 2). Based on the observations in our results we don't recommend the CRA method as a suitable method for detection of biofilm formation.

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

ACKNOWLEDGEMENTS

P. aeruginosa is an increasingly prevalent opportunistic pathogen, which causes chronic pneumonia patients and severe life-threatening infections in immunocompromised persons. In addition, *P. aeruginosa* readily assumes the biofilm life style, which confers efficient protection against the activity of the host defense system. It exhibits an inherent tolerance to many of the antibiotics most commonly used, which emphasizes the urgent need for development of novel strategies that will help us to defeat this pathogen.

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