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**Original Research Article**

# Forging a link between bacterial biofilms and drug resistance: an unsolved mystery

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## ABSTRACT

**Background:** Though use of various medical devices as indwelling catheters, cardiac pacemakers, prosthetic heart valves, chronic ambulatory peritoneal dialysis catheters, and prosthetic joints has greatly facilitated management of serious illness. Bacterial strategies to colonize and grow as biofilms on these devices are major cause of morbidity among patients receiving prosthesis.
**Methods:** Fifty *Pseudomonas aeruginosa* (*P. aeruginosa*) strains isolated from the urine samples of catheterized patients were subjected to biofilm detection by Tissue Culture Plate method and MIC of ciprofloxacin was determined against them using broth dilution method.
**Results:** In our study 50 (7.69%) *P. aeruginosa* isolates were subjected to biofilm screening by TCP. Among 50 isolates, TCP method detected 40 (80.00%) biofilm producers. Out of which 28/50 (56.00%) were high, 12/50 (24.00%) were moderate and 10/50 (20%) were non/weak biofilm producers. MIC for ciprofloxacin was detected for *P. aeruginosa* strains at various concentrations (0.25ug/ml -8ug/ml). We observed that the MIC range for high biofilm producing *P. aeruginosa* was between 4- 8ug/ml, whereas for non-biofilm producers MIC range varies from 0.25 to 1ug/ml. Thus, biofilm can pose a threat in patient treatment.
**Conclusions:** The armament of various bacteriostatic or bactericidal agents available to treat infections are restricted to act in planktonic phase and these agents did not take into account the unique biology of bacterial biofilms. Thus, bacteria growing as biofilm communities often result in troublesome complications as persistent infections, which cannot be resolved with standard antibiotic treatments. As, biofilm communities embedded in exopolysaccharide have not been considered until recently, therapeutic strategies to treat them are not available yet.

**Keywords:** Antibiotic resistance, Biofilms, Ciprofloxacin, *Pseudomonas aeruginosa*

## INTRODUCTION

Biofilm mode of growth deployed by microbial cells includes irreversibly association with surfaces and enclosed in a matrix of polysaccharide. Thus, bacterial biofilms are well organized, cooperating community of microorganisms.[1] Biofilm associated infections have major impact on permanent and temporary implants, often with devastating consequences. Moreover, they often serve as source for recurrent infections. Many persistent and chronic bacterial infections are believed to be linked to the formation of these sessile communities.[2] Researches estimated that 60-80 percent of microbial infections in the body are caused by bacteria growing as a biofilm as opposed to planktonic.[3] In human body bacterial biofilms provide protection against immune system and antibiotic treatment. Higher resistance among biofilm bacteria to antimicrobials is a serious issue and

reason for common therapy failure. Extracellular polysaccharide matrix plays key role in antibiotic resistance, as it prevents diffusion of drug into bacterial cells. It is the reason as higher concentration of antibiotic-reducing enzymes in the bacterial surroundings partakes in micro environmental changes in the deeper layers of biofilm. To add to this cascade, low pH reduces effect of some antibiotics (such as aminoglycosides) and the nutrition and oxygen deficiency leads to the growth stasis of bacteria (e.g. the beta-lactam antibiotics become ineffective). Thus, these physiological changes among bacteria embedded in biofilm layer account for ineffective treatment.[4]

This present study is undertaken to study the effectiveness of ciprofloxacin against biofilm producing strains of *Pseudomonas aeruginosa* (*P.aeruginosa*), as they are highly adherent to various biomaterial surfaces and are invariably recalcitrant to antimicrobial treatment because of virulence properties including slime production.

## METHODS

This observational prospective study was undertaken in the Department of Microbiology in a tertiary care institute. Urine samples from patients catheterized for >7 days, admitted in the various wards of hospital were taken after informed consent following aseptic precautions.

Samples were cultured on MacConkey agar and blood agar and incubated overnight aerobically at 370C. The organisms were identified by conventional microbiological techniques.[5] Total of 650 non-repetitive isolates namely *Escherichia coli* (n=232), *Klebsiella pneumonia* (n=108), *P. aeruginosa* (n=50), Proteus mirabilis (n=65), and *Staphylococcus spp*. (n=195) were isolated. Among the gram negative isolates, we selected *P. aeruginosa* as present study organism that was further screened for biofilm production and MIC for Ciprofloxacin was evaluated.

### *Detection of biofilm production*

*P. aeruginosa*, the study organism, was screened for biofilm production by using spectrophotometry based most widely used Tissue culture Plate (TCP) method described by Christensen et al.[6]

Isolated strains were inoculated in brain heart infusion with 2% sucrose and incubated for 18 hour at 37°C in stationary condition and diluted 1 in 100 with fresh medium.

Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates were filled with 0.2 ml aliquots of the diluted cultures and only broth served as control to check sterility and non-specific binding of media. The tissue culture plates were incubated for 24 hours at 37°C. After incubation content of each well was removed by gentle tapping. Wells were washed four times with 0.2 mL of phosphate buffer saline (PBS pH 7.2) to remove planktonic bacteria. Biofilms formed by adherent 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. Adherent cells usually formed biofilm and were uniformly stained with crystal violet. Optical densities (OD) of stained adherent bacteria were determined with a micro ELISA auto reader at wavelength of 570 nm. These OD values were considered as an index of bacteria adhering to surface and biofilm formation. Strains with OD value >0.240 were high biofilm producer, 0.120-0.240 were moderate and OD < 0.120 were non biofilm producer.[7] *P. aeruginosa* ATCC 27853 and 25923 were used as positive and negative controls to asses biofilm production respectively.

### *Detection of MIC for ciprofloxacin*

Flouroquinolones are broad spectrum agents which penetrate bacterial cell walls and inhibit DNA gyrase (bacterial topoisomerase II) activity, thus, rapidly killing susceptible organisms.

Class representative, Ciprofloxacin being highly effective, especially against members of the family Pseudomonads, providing a standard against which other compounds are compared.[8] Minimum inhibitory concentration (MIC) testing was performed in accordance with Clinical and Laboratory Standards Institute using broth dilution method.[9]

## RESULTS

*P. aeruginosa* (n=50) isolates were screened for biofilm production by TCP method which detected 40 (80.00%) among them as biofilm producers. 28/50 (56.00%) were high, 12/50 (24.00%) were moderate and 10/50(20%) isolates were non/weak biofilm producers. MIC of ciprofloxacin as determined by the broth dilution method at various concentration range (0.25ug/ml to 8ug/ml), results as shown in Table 1.

**Table 1: MIC values of *P. aeruginosa* isolates to ciprofloxacin.**

| MIC range (Ciprofloxacin) | <1ug /ml | 1ug /ml | 2ug/ ml | 4ug /ml | 8ug /ml |
|---|---|---|---|---|---|
| Total sensitive isolates (n=50) | 8 | 2 | 6 | 15 | 19 |
| Biofilm producer | nil | nil | 6 | 15 | 19 |

It was observed that the MIC value for P. aeruginosa non biofilm forming isolates was <1ug/ml, whereas the MIC value of high biofilm producers was varying between 4-8ug/ml (Figure 1).

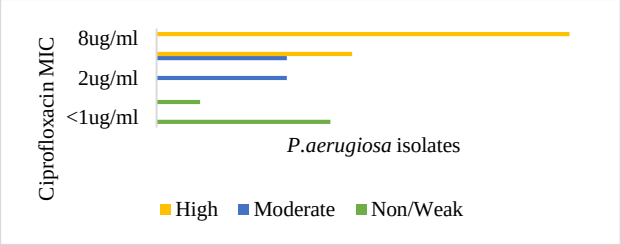


**Figure 1: Distribution of *P. aeruginosa* isolates Biofilm Vs Ciprofloxacin MIC.**

## DISCUSSION

In present study 50 (7.69%) *P. aeruginosa* isolates were subjected to biofilm screening by TCP. Among 50 isolates, TCP method detected 40 (80.00%) biofilm producers. 28/50 (56.00%) were high biofilm producers, 12/50 (24.00%) were moderate and 10/50(20%) isolates were non/weak biofilm producers. Present results are in agreement with other investigators with little variations as shown in Table 2.

**Table 2: *P. aeruginosa* isolates and biofilm production in other studies.**

| Author | Sample | Isolate | *Biofilm method | Biofilm producers |
|---|---|---|---|---|
| Charan KG et al[10] | urine | 21 (14.38%) | TM | 14 (66.66%) |
| Mushtak T Salih et al[11] | urine | 12 (16.00%) | TCP | 12 (100%) |
| Parmodhini S, et al[12] | urine | 4 (4%) | TM | 4 (100%) |
| Abdallah NMA et al[13] | urine | 5 (6.7%) | TCP | 3 (60%) |
| Nagaveni S, et al[14] | Pus/urine | 11 (44.00%) | TCP | 8 (72.72%) |

*TCP-Tissue culture plate method; *TM-Tube Method.

*P. aeruginosa* is an important opportunist pathogen and is eminent cause for chronic infections including urinary tract infections (UTI). This debilitating disease is a serious health problem affecting number of people worldwide and urinary tract catheterization is one of the most common predisposing factors to such infections. Indisputably, bacterial survival strategy as embedded in biofilm was a key feature of *P. aeruginosa* in chronic infections as these extracellular matrices provide structural scaffold and form protective barricade against antibiotics.[15]

Various risk factors have been studied to be associated with UTI. There are many studies that indicate that risk of UTI is related to duration of catheterization.[16-18] Johansen TE et al, Tambyah PA et al have indicated daily risk of bacteriuria increases considering catheter day cut-off as 3 days where as Langley JL et al and Lohr et al have suggested cut-off point to be 7 days.[16,17,19,20] Langley and co-workers, observed that infection rate was 95%, among patients catheterized >28 days.[19] This rate was 10 times higher after seventh day of catheterization. Therefore, we selected samples from duration of catheterization >7 days.

A production of extracellular polysaccharide matrix or glycocalyx prevents access of antibiotics to bacterial cell and plays the key role in recalcitrance of biofilms to antimicrobial treatment.[21] In *P. aeruginosa* biofilm, the efficacy of the antimicrobial agent may be attenuated not only by decreased accessibility to the sheltered bacteria caused by exopolysaccharide alginate matrix but also by emergence of mutant subpopulations.[15] *P. aeruginosa* isolates screened for biofilm formation by TCP method were further evaluated against ciprofloxacin MIC at various concentration range (0.25ug/ml -8ug/ml) as flouroquinolones are particularly potent in the urinary tract, making them an attractive choice for prophylaxis and treatment of urinary tract infection. Ciprofloxacin resistance has been closely associated with multi-drug resistance, it can be used as a surrogate marker for multi-drug resistance, therefore limits the already restricted treatment options.[22] We observed that the MIC range for high biofilm producing *P.aeruginosa* was between 4-8ug/ml, whereas for non-biofilm producers MIC range varies from 0.25 to 1ug/ml (Figure 1).

**Table 3: Ciprofloxacin resistance observed among biofilm producers in other studies.**

| Author | Sample | Ciprofloxacin resistance |
|---|---|---|
| Charan et al[10] | Urine | 60% |
| Nagaveni S, et al[14] | Pus/urine | 100% |

## CONCLUSION

Present study reinforces findings of other authors suggesting that biofilm makes bacteria more resistant to antibiotics, which in this case was ciprofloxacin, posing a threat in the patient treatment. A clear understanding of the role of biofilms in infection should guide the clinical decision making and also proper use of therapeutics.

There is no one answer to the question concerning bacterial biofilm formation and possible mechanisms that account for their increased resistance to antimicrobial compounds. It is clear that performing more extensive

studies focused on biofilm formation can elucidate how and why these sessile communities protect themselves and could indicate potential targets for their prevention and treatment of these infection.

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