Biofilm formation and associated antibiotic resistance in pathogenic *Pseudomonas aeruginosa*

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1 Introduction:

1.1 **Pseudomonas aeruginosa:** *P. aeruginosa* is an opportunistic pathogen, gram negative with short rod, non-sporulating, non-capsulated, motile by means of one or two polar flagella. WHO has listed *P. aeruginosa* as 3rd critical priority list pathogens.

1.2 **Epidemiology:** *P. aeruginosa* which is adapted to form biofilm on surface and causes catheter-associated urinary tract infection (CAUTI), ventilator-associated pneumonia, infections related to mechanical heart valves, stents, grafts, and sutures, and contact lens-associated corneal infections. Catheter-Associated Urinary Tract Infections (CAUTIs) contribute significantly to hospital associated infections (HAIs) (Lara-Isla et al. 2017), which are often persistent due to infections caused by biofilm forming bacteria. *P. aeruginosa* is the third most common pathogen associated with hospital acquired CAUTIs (Jarvis and Martone 1992; Djordjevic et al. 2013). The antibiotic used to treat biofilm infection may decrease the load of bacteria within the biofilm, however cannot completely eradicate the biofilm (Lamas Ferreiro et al. 2017), and thus biofilm infection reoccurs.

1.3 What is biofilm?

Biofilms are generally known as communities of microbes that are attached to certain surfaces that are normally covered with an extracellular matrix (ECM), secreted by the same microbes. ECM mainly consists of polysaccharides, nucleic acids (extracellular DNA) and proteins, which helps to protect the micro-organisms from external threats, including immune system components and antimicrobials. (Percival et al. 2010). Biofilms are medically important because they are involved in the pathogenesis of numerous bacterial infections that are difficult to successfully eradicate with antibiotics (Costerton et al. 1999).

1.4 ***P. aeruginosa* biofilm:** *P. aeruginosa* biofilms have distinct developmental stages. The initial attachment of *P. aeruginosa* is mediated by adhesins, type IV pili, Psl, and lipopolysaccharides, and is regulated by c-di-GMP and small regulatory RNA (sRNA). (Lee and Yoon 2017) The developmental stages of *P. aeruginosa* biofilm as following: (1) the planktonic stage, (2) attachment of bacteria to a surface, (3) production of the extracellular matrix, (4) maturation of biofilm structures, (5) spatial differentiation, and (6) biofilm dispersal.

***P. aeruginosa* biofilm components:** The biofilm comprises an extracellular matrix having polysaccharides (alginate, pel, and psl), proteinaceous components (type 4 pili, CdrA adhesins, cub fimbria, lecAB lectins), and extracellular DNA (eDNA) (Vallet et al. 2001; Klausen et al. 2003; Tielen et al. 2005; Borlee et al. 2010; Mulcahy et al. 2014; Thi et al. 2020).

1.5 Antibiotic resistance mechanism in *P. aeruginosa* biofilm:

1.6 Antibiotic resistance generally means an increase in the minimum inhibitory concentration (MIC) value of an antibiotic due to a permanent change in the bacteria, e. g., mutation or resistance acquired through the horizontal gene transfer. In biofilm state bacteria are 10 to 100 times more resistant than the planktonic form. There is probably no single mechanism that accounts for biofilm tolerance or resistance.

The major antimicrobial resistance and tolerance mechanism employed by *P. aeruginosa* biofilms: (1) nutrient gradient with less nutrient availability in the core of the biofilm, (2) matrix exopolysaccharides, (3) extracellular DNA, (4) stress responses (oxidative stress response, stringent response, etc.), (5) discrete genetic determinants that are specifically expressed in biofilms and whose gene products act to reduce biofilm susceptibility via diverse mechanisms (*ndvB, brlR*, etc.), (6) multidrug efflux pumps, (7) intercellular interactions (Hall and Mah 2017).

Persister cell: Persisters are defined as subpopulations of cells, occurring at very low frequency, which stochastically emerge in the presence of stress. Increased levels of (p) ppGpp (collectively designated for guanosine pentaphosphate and guanosine tetraphosphate) molecules in the cells is a central triggering alarmone for both persistence and stringent response. The cellular levels of (p) ppGpp are mediated by the activity of the (p) ppGpp synthesizing and degrading enzymes such as RelA and SpoT in response to external stimuli. To date required elements of stringent response and persistence including the ppGpp alarome, SpoT, rel A, DskA, carB, and TA modules have been characterized in *P. aeruginosa.*

2 Rationale:

Catheter-associated urinary tract infections (CAUTIs) are one of the major problems in a hospital environment. Among which 75 % are associated with urinary tract infection in hospital-acquired UTI. *P. aeruginosa* is one of the major opportunistic pathogens that is resistant to many antibiotics and responsible for the CAUTIs. Many studies have been conducted on CF patients isolates while there are limited studies related to the UTI isolates. Biofilm variation in strong and weak biofilm producers in context to components and biofilm formation. Apart from that, the persister cell formation is much highlighted in *Escherichia coli* than *P. aeruginosa*. Also, persister cell formation mechanism in biofilm with regard to different antibiotic is less understood. There is need to study persister cell formation with regards to planktonic and biofilm stage with different antibiotics.

3 Objectives

1) Collection, isolation and identification of clinical isolates of pathogenic *Pseudomonas aeruginosa.*
2) Characterization of biofilm components
3) Antibiotic resistance in biofilms

4 Results:

4.1 Work done:

Objective 1: Collection, isolation and identification of clinical isolates (n=22) of UTIs *Pseudomonas aeruginosa* were collected from Toprani and sterling lab, Vadodara, Gujarat India. The following isolates were Gram stained and found to be Gram negative bacteria. Also, the isolated isolates were grown on Pseudomonas isolation agar (PIA) which is specific for *P. aeruginosa.* Further, 16 sRNA sequencing was performed for following isolates and all were found to be *P. aeruginosa* (n=22).

Objective 2: Characterization of biofilm components

P. aeruginosa clinical isolates (n=22) causing UTI and PAO1(used as reference strain) were classified as strong, moderate and weak biofilm producers using biofilm crystal violet assay (Stepanović et al. 2004). Majority of the isolate were found to strong (n=16) followed by moderate (n=2) and weak (n=4) biofilm producers. For further study following isolate of strong (ST-20, TP-25, TP-35 and TP-48) and weak (ST-22, TP-8, TP-10 and TP-11) biofilm producers were selected randomly. Growth curve analysis was performed and no significant difference was found between strong and weak biofilm producers. The mean growth rate of both (strong and weak biofilm producers) was 0.24 ± 0.02 per hour. Biofilm quantification was done on silicone-coated latex and silicone catheters. The amount of biofilm formation was observed in presence of LB, NU and AU in both (strong and weak biofilm producers). On both catheters there was no difference in biofilm formation irrespective of medium in strong biofilm producers. Whereas, in weak biofilm producers the highest biofilm formation was observed on silicone-coated latex catheter compare to silicone catheters. Strong biofilm producers formed highest biofilm formation compared on silicone catheters in LB compared to weak biofilm producers.

The adhesion ability of strong was more than weak biofilm producers using light microscopy. The number adhered cells in strong (n=727 ± 100) was significantly more than weak (n=127 ± 29) biofilm producers. Type 4 pili (T4P) mediated twitching motility is essential for surface attachment and the initial stage of microcolony formation. So, we next observed the twitching motility of strong and weak biofilm producers. On 1% agar plate there was significant difference in twitching motility zone of strong (1.1 ± 0.40 mm) and weak (0.6 ± 0.21 mm) biofilm producers. Phase Contrast Time Lapse- Microscopy was done at 4 hours, where the number of twitching cells were more in strong (n=4) compare to weak biofilm producers. Off note, one of the isolate TP-10 lacked twitching motility. As a result of high twitching in strong biofilm producers, wrinkly colony edge formation was observed in strong biofilm producers after 24 hours of incubation. Whereas, in weak biofilm producers no fully wrinkly colony edge formation was observed. The quantification of gene expression for the T4P gene was done at 4 h and 24 h biofilms. The type 4 pili expression was 5-fold and 3-fold more in strong than weak biofilm producers at 4 hours and 24 hours.

The difference in strong and weak biofilm producers was visualized by Confocal Laser Scanning Microscopy (CLSM) using Syto 9 (stains live cells) and PI (dead cells) dyes. Within the orthogonal plane of biofilm formed by strong biofilm producers has greater number of dead (red) cells than live cells (green). However, in weak biofilm producer's biofilm the number of live cells were more than dead cells. Further, we also observed variation in thickness of strong (31.25 µm ± 14.3) and weak (19.05 µm ± 9) biofilm producers. The Z-stack videos of biofilm gives more clear idea about the arrangements of live and dead cells within the biofilm. The strong biofilm producer's biofilm has more number dead cells near substratum and live cells above them (cells were densely packed). However, in weak biofilm producer's biofilm the number of live were more near substratum and dead cells were less (cells loosely packed). Field Emission Gun- Scanning Electron Microscopy (FEG-SEM) was done to study biofilm on

silicone-coated latex catheters. The magnification at 3000X, 6000X and 12000X shows that biofilm formed by strong biofilm producers has thick biofilm and bacterial cells ad embedded within exo-polymeric substance (EPS). While in weak biofilm producer's biofilm only cells were found to adhere to each other and catheter and microcolony formation was observed.

Also, quantification of biofilm component (eDNA, extracellular protein, pel, alginate, rhamnolipid and pyocyanin) was done in strong and weak biofilm producers. The amount eDNA, extracellular protein and pel polysaccharide was high in strong compared to weak biofilm producers. There no difference in gene expression of cdr A gene within biofilm formed by strong and weak biofilm producers.

Effect of exogenous treatment (enzymes, eDNA and protein) was checked on biofilm formation of strong and weak biofilm producers. Effect of enzymatic treatments (DNase I, proteinase K, and RNase) on biofilm formation by strong biofilm producers showed the highest inhibition by proteinase K treatment, followed by RNase and DNase treatment that reduced the biofilm by 76.35%, 63.43%, and 43.35 %. Out of the three treatments, only DNase treatment resulted in a significant reduction of biofilm (58.27%) in weak biofilm producers. Further, we hypothesized that the addition of exogenous DNA and extracellular protein may increase the amount of biofilm. A decrease in biofilm was observed in both strong and weak biofilm producers upon the addition of eDNA when compared to control (no eDNA is added). No significant difference was observed on addition of extracellular protein in comparison to control (no extracellular protein is added).

Objective 3: Antibiotic resistance in biofilms

3.1 Effect of Antibiotic in persister cell formation

For persister cell formation study following isolates PAO1 (used as reference strain), TP-10 and ST-13 of *P. aeruginosa* were selected. The Minimum Inhibitory concentration (MIC) was

carried out using CLSI guideline for following antibiotics: ceftazidime, gentamicin and ciprofloxacin. The table 1 shows the MIC results. Further time kill curve assay was done for n=3 isolates in planktonic stage. The time kill curve assay was done for t=1, 2, 4, 6, 8 and 24 hours. The kill curve assay result shows that ceftazidime has high fraction of persister cells compare to gentamicin and ciprofloxacin treatment at 4 hours in PAO1 and ST-13 isolates. while, in TP-10 isolates gentamicin and ciprofloxacin showed low fraction of persister cells compared to ceftazidime.

Table 1: MIC results

Sample	MIC (mg/L)		
	CAZ	GEN	CIP
PAO1	1 (S)	0.5 (S)	8 (R)
TP-10	0.5 (S)	0.5 (S)	0.0625 (S)
ST-13	2 (S)	1 (S)	8 (R)

Flow cytometry was done to quantify the abundance of persister cells formation in planktonic stage of TP-10 and ST-13. Quantification of persister cell was done using RSG and PI dyes after 4 hours of antibiotic treatment. The RSG dye can easily penetrate bacteria and yield green fluorescence when reduce by bacterial reductase. The quantification was done after 4 hours in the regime of biphasic pattern of kill curve to ensure susceptible cells are not measured and persister cells are measured. In TP-10 isolate there was significant difference in persister cell (RSG stained cells) between growth control (untreated with antibiotic) and antibiotic treatment (gentamicin and ciprofloxacin) except for ceftazidime antibiotic treatment. However, there was

no difference in persister cells was observed between growth control and antibiotic treated (ceftazidime, gentamicin and ciprofloxacin).

To further gain insights into *P. aeruginosa* persistence upon ceftazidime, gentamicin and ciprofloxacin treatment, following genes responsible for persister cell formation (1) three stringent response genes (*spoT, relA and lon*), and (2) two genes of HigB-HigA toxin-antitoxin system (*higA and hibB*). All genes were studied at 4 hours of antibiotic treatment at 5X MIC concentration and compared with untreated control. In PAO1 isolate, compared to untreated control gentamicin treatment showed 5.1-,10-,10-, 65-,1.3-fold change gene expression in *relA, spoT, higA, higB* and *lon.* Followed by ciprofloxacin (8.8-, 8-, 6.4-, 2.3- and 0.2-fold change) and ceftazidime (1.9-, 2-, 2.5-, 2-, and 0.8-fold change). In TP-10 isolate, compared to control in variation in gene expression of persister cell was observed as 1.5-,2.5-,6.5-, 2.8- and 1-fold change in ciprofloxacin; 0.3-,1.2-,1.5-,366.56- and 0.7- fold change. Whereas upon ciprofloxacin treatment 0.9-, 3.4-,4.2-,23- and 0.5-fold change in *relA, spoT, higA, higB* and *lon* gene expression was found.

Persister cells formation was also studied in biofilm stage with above isolates (n=3). The biofilm forming ability was checked using crystal violet assay. Where PAO1 and ST-13 were among strong biofilm producer, whereas TP-10 was weak biofilm producers. Further the effect of at 5X MIC concentration antibiotic treatment (ceftazidime, gentamicin and ciprofloxacin) on biofilm was studied using resazurin dye (stains metabolically active cells). The maximum reduction in metabolically active cells was observed in ciprofloxacin treatment in PAO1. Also, in TP-10 isolate, maximum reduction was observed in ciprofloxacin followed by gentamicin. However, in ST-13 isolate the reduction in metabolically active cells was observed in all three antibiotic treatments. Time kill curve analysis was also done with 5X MIC antibiotic concentration (ceftazidime, gentamicin and ciprofloxacin). In all isolate, ceftazidime showed least effective in inhibition and regrowth of biofilm was observed. The biphasic killing pattern

was observed in gentamicin and ciprofloxacin. Where lowest fraction of persister cell was observed in ciprofloxacin treatment followed by gentamicin compared to growth control.

Flow cytometry was done to analyze the persister cell formation in biofilm stage after antibiotic treatment. There was no significant difference observed in RSG fluorescence in TP-10 and ST-13 biofilm. The gene expression studies for stringent response (*relA*, *spoT* and *lon*) and toxin-antitoxin (*higA* and *higB)* was done after 4 hours of antibiotic treatment at 5XMIC concentration. The gene expression was observed to upregulated in gentamicin and ciprofloxacin exposure. The gene expression of *relA*, *spoT*, *lon*, *higA* and *higB* gene on gentamicin treatment were: 1.27-,2.3-, 5.6-, 2.7-, and 2.7-fold change and ciprofloxacin treatment resulted: 1.51-, 3.4-, 5.1-, 0.9-, and 3.1-fold change compared to untreated control in PAO1 isolate. No significant increase in gene expression was found in TP-10 biofilm. However, in ST-13 biofilm, the gene expression in gentamicin was: 4.8-, 1.9-, 21-, 10.5-, 10-fold change and on ciprofloxacin treatment: 2.7-, 0.9-, 2.5-, 4.3-, 5.6- fold change in gene expression of *relA*, *spoT*, *lon*, *higA* and *higB.* On ceftazidime treatment there was no upregulation of stringent as well as toxin-antitoxin genes compared to untreated in all isolates.

5 Summary

The present study was done to understand the biofilm formation in UTI causing *P. aeruginosa* isolates. The *P. aeruginosa* isolates were categorized as strong, moderate and weak biofilm producers. Most of the isolates were found to strong biofilm producers followed by weak and moderate. Diverseness in biofilm formation was observed between strong and weak biofilm producers on catheters. On silicone-coated latex catheters more biofilm formation was observed in strong and weak irrespective of medium (LB, NU, and AU) used compared to silicone catheters. Further, cell adhesion ability, twitching motility, pel polysaccharide, dead cells and total (dead and live cells) contribute to biofilm formation in strong biofilm producers.

Many cell death pathways have been reported in PAO1 isolates such as prophage mediated, autolysis, eDNA release and many more which could contribute to cell death in strong biofilm producers. Apart from this, biofilm resistance in one of problem of biofilm relation infection which can lead to relapse of infection. On of mechanism in biofilm resistance is persister cell formation and relapse of biofilm infection. We found on gentamicin treatment stringent response as well as toxin-antitoxin genes were upregulated followed by on ciprofloxacin exposure and ceftazidime exposure in planktonic stage. Whereas in biofilm stage ceftazidime was least effective on biofilm inhibition and regrowth was observed. Persister cell formation was observed on gentamicin and ciprofloxacin treatment on biofilm. It was also observed persister cell formation varied across isolates on different antibiotic treatments.

6 References

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