SUMMARY

Objective 1: Collection, isolation, and identification of clinical isolates of pathogenic *P. aeruginosa*

- Gram-negative clinical isolates (n=22) from patients with UTIs were collected from Sterling and Toprani Lab, Vadodara, Gujarat, India.
- The collected isolates were subcultured on a selective medium known as Pseudomonas Isolation Agar containing triclosan (irgasan) that inhibits the other Gram-negative bacteria as well as other *Pseudomonas spp.* The round with raised elevation colonies were observed and green pigment was diffused into the medium. Some of the isolates also produced blue-green pigment (pyocyanin) and were diffused in the medium.
- The Gram staining was performed on the isolated colonies grown on PIA. All of the isolates (n=22) were found to be Gram-negative bacteria.
- All of the above isolates were further identified through molecular identification. The genomic DNA was isolated from n=22 isolates and the 16S *rRNA* gene was amplified using PCR. Then, the amplified product was sent for Sanger sequencing. Using the NCBI BLAST tool all hits (n=22) were found to be of genus *P. aeruginosa.* For further study PAO1 and n=22 isolates were used.

Objective 2: Quantification and characterization of biofilm components

- Biofilm forming ability of n=22 clinical isolates *P. aeruginosa* causing UTIs was checked on a 96-well plate using crystal violet assay and further categorized into strong moderate and weak biofilm producers. The majority of the isolates were identified as strong (n=16), while few were moderate (n=2), and weak (n=4) biofilm producers. From the above biofilm producers following isolates of strong (ST-20, TP-25, TP-35and TP-48) and weak (ST-22, TP-8, TP-10, TP-11) biofilm producers were randomly selected for further studies.
- Biofilm quantification was also done on various catheters (silicone-coated latex and silicone catheters) in presence of different media. The highest biofilm formation by strong and weak biofilm producers was observed on LB followed by NU and AU (* $P < 0.05$, ** $P < 0.005$) (Figure 4.2 a, and b). Further, a negligible difference in biofilm formation was observed by strong biofilm producers on silicone-coated latex catheters compared to silicone catheters irrespective of the medium used (* $P < 0.05$) (Figure 4.2 c). However, in presence of LB medium highest biofilm formation was observed by

weak biofilm producers on silicone-coated latex catheters than silicone catheters (* $P < 0.05$) (Figure 4.2 d), and no difference was observed in presence of NU/AU.

- The environmental mode FEG-SEM was performed for biofilm formed by strong (TP-25) and weak (TP-8) biofilm producers on silicone-coated latex catheters. The thick biofilm, rod-shaped cells encased within the dense EPS matrix was observed in strong biofilm producers on the magnification at 3000X,6000X, and 12000X (Figure 4.3a; upper panel). On the other hand, a thin layer of bacteria adhered to the catheter as well as each other was observed in weak biofilm producers (Figure 4.3b; lower panel).
- Further differences in strong and weak biofilm producers were studied using adhesion ability on a coverslip in a 6-well plate followed by Gram-staining. The adhesion ability of strong biofilm producers was high compared to weak biofilm producers (Figure 4.4a). Also, the number of adhering cells was high in number in strong ($n=727 \pm 100$) than in weak ($n=127 \pm 29$) biofilm producers (Figure 4.3b).
- In addition, the twitching motility and twitching zone of strong biofilm producers (1.1 ± 0.40 mm) was significantly higher than weak (0.6 ± 0.21 mm) biofilm producers on 1% agar plate (*** $P < 0.005$) (Figure 4.5a and c). Through time-lapse Phase Contrast Microscopy, the twitching motility was studied on a 1% LA pad. The twitching cells on the colony edge after 4 hours were observed to be high in strong than weak biofilm producers (Suplementary video S1 from: https://doi.org/10.1080/08927014.2022.2054703) and wrinkly colony edge formation was observed in strong producers compared to weak (Figure 4.5 b). The gene expression of the *pilA* gene was studied after 4 hours and 24 hours. The *pilA* gene was 5-fold and 3-fold significantly high in strong at 4 hours and 24 hours than weak biofilm producers (* $P < 0.5$, ** $P < 0.05$) (Figure 4.5d).
- Further visualization of strong and weak biofilm producer's biofilm was observed through CLSM using Syto9 (stains live cells in green colour) and PI (stains dead cells in red colour). Through orthogonal plane visualization, the strong biofilm producers had a greater number of dead cells and a small number of live cells (Figure 4.6a). Whereas in weak biofilm producers live cells were more than dead cells except in the TP-11 isolate where dead cells were high in number due to pyocyanin production as observed in Figure 4.6c. The difference in biofilm thickness was significantly observed to be 31.25 µm ± 14.3 in strong and 19.05 µm ± 9 in weak biofilm producers (** $P < 0.05$) (Figures 4.6 a and c).

- The strongly packed wells were observed in strong biofilm producers (Figure 4.6b) and loosely packed cells were observed near the substratum in weak biofilm producers (Figure 4.6d). The cells were densely packed, dead cells were beneath the live cells of the substratum in strong however loosely packed live cells and a sparse number of cells were observed near the substratum of weak biofilm producers observed in the video file (supplementary video S2: https://doi.org/10.1080/08927014.2022.2054703). From the Z stack analysis of each strong (n=4) and weak (n=4), biofilm formed strong and weak biofilm producers showed a significant difference in cell count (total cells and dead cells) (* $P < 0.5$, ** $P < 0.05$) (Figure 4.6e).
- The following components were significantly high in strong biofilm producers: eDNA (* $P < 0.05$), extracellular protein (** $P < 0.005$), and pel polysaccharide (* $P < 0.05$) in comparison to weak biofilm producers (Figure 4.7 a-c). There was no difference observed in the alginate polysaccharide, rhamnolipid, and pyocyanin production (Figures 4.7 c, and d).
- The effect of the exogenous addition of DNase, RNase, and proteinase K was studied on biofilm formed by strong and weak biofilm producers. There was significant reduction was observed in DNase (* $P < 0.05$), RNase (** $P < 0.005$), and proteinase K (*** $P < 0.0005$) treatment on biofilm formed by strong biofilm producers (Figure 4.8a). While weak biofilm producers on DNase treatment (* $P < 0.05$) showed a significant reduction in biofilm formation (Figure 4.8a).
- Further, with the addition of gDNA (* $P < 0.05$) and eDNA (* $P < 0.05$), there was a significant reduction in biofilm formation by strong biofilm producers, and no difference was observed in weak biofilm producers.

Objective 3: Antibiotic resistance in biofilm

a. To study the effect of antibiotics in persister cell formation

- The PC formation was studied in n=2 isolate (ST-13 and TP-10) and PAO1 was used as a reference strain. The PC formation was studied in three different classes of antibiotics: ceftazidime (cell wall inhibiting), gentamicin (protein synthesis inhibitor), and ciprofloxacin (DNA gyrase inhibitor). The MIC was done for all isolates along with PAO1 for the following antibiotics: ceftazidime, gentamicin, and ciprofloxacin using CLSI guidelines. The PAO1 and ST-13 were resistant to ciprofloxacin while susceptible to gentamicin and ceftazidime antibiotics. The TP-10 isolate was susceptible to all antibiotics.

- The time-kill curve assay was performed for all three isolates at 5X MIC level of antibiotics for 24 hours at indicated time points. The biphasic kill curve varied across the isolates. On ceftazidime treatment, the least log cfu/ml reduction was observed in all three isolates compared to the growth control (Figure 5.1a-c). While there was a variation of log cfu/ml in gentamicin and ciprofloxacin treatment in all isolates (Figure 5.1a-c).
- Further, the cellular redox activity was checked using flow cytometry after 4 hours of 5X MIC level of antibiotic treatments. To study the redox activity of the cells the cells were stained with RSG (metabolic indicator dye) and PI (stains dead cells in red colour) dyes. The ceftazidime treatment had high redox activity in all three isolates compared to the growth control (Figure 5.2a-c). While the antibiotic treatments of gentamicin and ciprofloxacin varied across the isolates having low redox activity (Figure 5.2a-c). The quantitative data of RSG-positive cells significant increase in cellular redox activity in PAO1 and TP-10 isolate after ceftazidime treatment compared to growth control (Figure 5.2 d, and e). While the significant decrease in redox activity was observed in gentamicin and ciprofloxacin treatment compared to growth control (Figure 5.2d). And no difference was observed in TP-10 isolate on gentamicin and ciprofloxacin treatment. No difference was observed in ST-13 isolate.

  The variation in redox activity observed in all isolates might be due to variation in log cfu/ml data among the isolates.
- Microscopy of PC formation was studied at 5X MIC level of antibiotic treatment after 4 hours and staining with RSG and PI dyes in the planktonic stage. On ceftazidime treatment elongation of cells was observed on all three isolates, however, the number of redox-active cells was observed in PAO1 and TP-10 isolates and less number in ST-13 isolate (Figure 5.3 b, f, and j). On gentamicin treatment, rod-shaped redox active cells were observed in three isolates. Ciprofloxacin treatment leads to a greater number of dead and fewer redox-active cells.
- The gene expression of stringent response (*relA*, *spoT*, and *lon*), as well as toxin-antitoxin (higA, and higB), was studied. The gene expression of *relA*, *spoT*, and *lon* varied across the isolates on various antibiotic treatments (Figure 5.4 a-c). While there was significant expression of *higA* and *higB* on gentamicin and ceftazidime in PAO1 and TP-10 isolates (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$) (Figure 5.4 d,

and e). While in ST-13 only *higA* was upregulated on gentamicin treatment (****P < 0.0001) (Figure 5.4 f).

- PC formation was also studied in the biofilm stage in TP-10, ST-13 and PAO1. Where ST-13 and PAO1 were strong biofilm producers while TP-10 was weak biofilm producer (Figure 5.5). The biphasic kill curve was performed after the biofilm was formed for 24 hours and treated with respective antibiotics. On ceftazidime treatment, the biofilm was regrown as untreated control (Figure 5.6a-c). The biphasic kill was observed for gentamicin and ciprofloxacin treatment and varied across the biofilm (Figure 5.6a-c).
- The redox activity was measured using flow cytometry where ceftazidime treatment led to an increase in redox activity followed by gentamicin and ciprofloxacin in PAO1 and TP-10 biofilm and not in ST-13 biofilm compared to untreated control (Figure 5.7 a-c). However, the quantitative analysis of RSG-positive cells showed a significant increase in redox activity on ceftazidime treatment only of PAO1 (Figure 5.7 d). And no difference in redox activity was observed for TP-10 and ST-13 isolates (Figure 5.7 e, and f).
- The CLSM was used to study the PC formation in the biofilm stage after respective antibiotic treatment and staining RSG and PI dyes. The untreated images of biofilm show variation in thickness across the isolate. Further ceftazidime treatment elongation of cells was observed in PAO1 and TP-10 isolates and having a smaller number of redox-active cells (Figure 5.8 b, and f). While in ST-13 biofilm the elongation of cells was less observed (Figure 5.8 j). Gentamicin treatment had rod-shaped redox active cells in PAO1, TP-10, and ST-13 biofilm (Figure 5.8 c, g, and k). While ciprofloxacin treatment leads to disruption of biofilm and dead cells were observed in all isolates (Figure 5.8 d, h, and l). The redox-active cells are less in number across the isolate
- The gene expression of stringent response as toxin-antitoxin varied across the isolates. The stringent response genes varied across the biofilm formed by PAO1, TP-10 and St-13 on different antibiotic treatments (Figure 5.9 a-c). No upregulation of *higA* and *higB* was observed in PAO1 biofilm on respective antibiotic treatments (Figure 5.9 d, and e). While *higB* and *higA* gene expression was observed in ST-13 biofilm on all three antibiotic treatments compared to untreated (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001) (Figure 5.9 d, and e).

b. <u>To study the biofilm-mediated antibiotic resistance through *brlR*</u>

- The whole genome sequence analysis was done for TP-11 and TP-10 isolates. Through WGS analysis *brlR, mexA, mexB, nalC, mexE, nalD* gene had substitution mutation in these genes (Table 5.11) and *sagS* gene was truncated from 1110-1560 position in DNA sequence of TP-10 isolate (Figure 5.10). while in TP-11 isolate *sagS, brlR, mexA, mexB, nalC, mexE* were present had substation in amino acid as shown in Table 5.11 and *nalD* gene had 2 bp deletion mutation as shown in Figure 5.11. PAO1 was used as a reference strain for this study.
- Next MIC for determined for PAO1, TP-10, and TP-11 isolates. POA1 was susceptible to gentamicin and resistant to ciprofloxacin antibiotics. TP-10 isolate was susceptible to both antibiotics. while TP-10 was resistant to both antibiotics.
- Biofilm formation was studied in a 96-well plate using CV assay. The PAO1 was a strong biofilm producer while TP-10 and TP11 were found to be weak biofilm producers (**$p<0.01$ and ***$p<0.001$) (Figure 5.12).
- There was no difference observed in the biofilm susceptibility assay (Figure 5.13). Further, pyocyanin production was significantly high in TP-11 biofilm in treatment with gentamicin and ciprofloxacin compared to untreated (Figure 5.14).
- Gene expression of *sagS, brlR, mexA, mexF* and *nalC* were upregulated in TP-11 isolate in compared to TP-10 isolate (Δ*sagS*) in untreated as well as antibiotics treated (Figure 5.15 a,b,c,d, and f). No upregulation of *nalD* was observed (Figure5.15 e). While in PAO1 no increase in transcript level of *sagS* gene was observed. Gene expression of *brlR* level were high in ciprofloxacin treatment only and gene expression of *mexA* and *mexF* as well as *nalD* gene (one of the negative regulators) was observed.

CONCLUSION

The present study aimed to isolate and identify the clinical isolates of *P. aeruginosa* from patient suffering from UTIs. Along with study of biofilm formation and biofilm-mediated antibiotic resistance against different antibiotics.

Isolation and identification of *P. aeruginosa* based on phenotypic methods were done on PIA agar and biochemical tests. This test might give a false positive result as many *P. aeruginosa* isolates may/ may not produce green/blue-green pigmentation on PIA. This was also observed in our data where not all isolates produced the green/ blue-green pigment as well as variation in pigment formation was observed. Thus, molecular identification by *16S rRNA* gene sequencing can be used for the detection of *P. aeruginosa* species level. In the present study, all isolates (n=22) were identified as *P. aeruginosa* species level.

Study of biofilm formation was studied in all collected clinical isolates of *P. aeruginosa* causing UTIs in patients. The majority of the isolates were strong biofilm producers followed by weak and moderate. Silicone-coated latex catheters have more biofilm formation compared to silicone catheters. So, the use of silicone catheters in the hospital setting can reduce the risk of biofilm-mediated infection/ CAUTIs. Diverse behavior was observed on catheters due to adherence ability, surface, cell appendages, and many more. Further, cell adhesion ability, twitching motility through *pilA* gene expression, and phase contrast microscopy were found to be high in strong biofilm producers. The adhesion ability can be exploited in the future to inhibit biofilm formation. Apart from these; eDNA, an extracellular protein, pel polysaccharide, the high number of total cells (dead and live) and dead cells contribute to biofilm formation by strong biofilm producers. From these, it can be said that high adhesion ability, twitching motility, eDNA, the dead, and the total number of cells are associated with biofilm formation by strong biofilm producers.

An important aspect of this study was to study the biofilm-mediated resistance in *P. aeruginosa*. The major concern in clinical areas is to eradicate biofilm-mediated infections as they are highly resistant to antibiotic treatments. One such mechanism majorly studied is PC formation. These PCs are often dormant and tolerant to antibiotic treatments and cause a relapse of biofilm-mediated infections. In this study, clinical isolates were also considered apart from PAO1 (as reference strain). The biphasic kill curve and redox activity within the planktonic stage of isolates varied across the antibiotic treatments. The important observation was the elongation of some PCs on ceftazidime treatments and rod-shaped formation on gentamicin/

ciprofloxacin treatments. This shows the heterogeneity in PC formation among isolates. Further, stringent response genes (*relA, spoT,* and *lon*) expression variation among isolates on different antibiotic treatments. However, there was the expression of toxin-antitoxin genes (*higB* and *higA*) on gentamicin and ceftazidime treatment in PAO1 and TP-10 isolate only. This demonstrates that persistence depends on the variability of the isolates as well as the growth phase and the type of antibiotics being used. Further biofilm formation also varied across the isolates and due to which the mean inoculum varied. This variation was also observed in the biofilm kill curve, and redox activity due to biofilm thickness/ biofilm cells. Microscopy gave more clear visualization of the variation of biofilm thickness across the isolates. Apart more this antibiotic treatment of ceftazidime leads to elongation of PC in PAO1 and TP-10 but redox active cells were less observed. While gentamicin and ciprofloxacin treatment had rod-shaped redox active cells. Further ciprofloxacin had more disruption of biomass and more number dead cells. Though the biofilm was disrupted the biofilm still had PC cells which may responsible for the relapse of the biofilm-mediated infection. The gene expression of stringent response varied across the isolates among the different antibiotic exposure. And toxin-antitoxin genes (*higB* and *higA*) were upregulated in ST-13 isolate on different antibiotic treatments. While no differential expression was observed in other isolates on different antibiotic treatments. From this finding, we hypothesize that the degree of antibiotic exposure and penetration within the biofilm, as well as the formation of the biofilm, are all factors that may affect PC formation in the biofilm stage. Overall findings suggest that compared to other antibiotic treatments, ceftazidime treatment causes PC development in the planktonic stage. However, in the biofilm stage, gentamicin and ciprofloxacin treatments lead to PC formation. Another biofilm-mediated resistance mechanism is through *brlR* which is biofilm specific. Isolates T10 (susceptible) and TP11 (resistant) were studied with respect to *brlR*. Increased antibiotic resistance in TP11 biofilm is attributed to multiple factors (mutated NalD, overexpression of *sagS* and *brlR*) causing over-expression of efflux pumps. Further studies are warranted to understand such strain specific differences in clinical isolates of *P. aeruginosa.*