ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen that is most commonly associated with hospital-acquired infections (HAIs). One of the HAIs is Urinary tract infections (UTIs) which are frequently caused by *P. aeruginosa* and is associated with high morbidity and mortality rate. Often this *P. aeruginosa* isolated from such infections are resistant to multiple antibiotics as well as found to form biofilms causing Catheter Associated-UTIs (CAUTIs). The infection caused by *P. aeruginosa* are often multidrug-resistant and listed as one of the priority pathogens by the CDC and WHO. *P. aeruginosa* often causes chronic infection by forming biofilm and often biofilm-related infections are difficult to treat. Biofilms are more resistant to antibiotic therapy than their planktonic counterparts. These infections are caused by persister cell (PC) formation or through gene-specific activation leading to drug tolerance of biofilm. Further, many studies are highlighted with respect to cystic fibrosis (CF) infections and scanty information is available regarding the UTIs specific clinical *P. aeruginosa* isolates. Also, the information regarding the PC formation with respect to the different antibiotic treatments at the supra-MIC level in the planktonic and biofilm stages is not understood. This study focuses on the biofilm-forming abilities of UTIs causing *P. aeruginosa* isolates. Along with that, biofilm resistance mechanism through (i) persister cell formation in planktonic and biofilm stage, and (ii) biofilm resistance mechanism through biofilm resistance locus regulator (*brlR*) gene in the biofilm stage was studied

Gram-negative clinical isolates (n=22) causing UTIs in patients were collected from Vadodara, Gujarat, India. After growing on Pseudomonas Isolation Agar (PIA), gram staining and molecular identification through 16s rRNA gene sequencing n=22 isolates were identified as *P. aeruginosa.* These isolates were further proceeded for biofilm and biofilm-mediated resistance studies.

The biofilm-forming ability of all clinical isolates (n=22) were determined and PAO1 was used as the reference strain. The biofilm was studied using crystal violet assay and further categorized into strong moderate and weak. Further studies were done with strong and weak biofilm producers on various catheters in the presence of different media. Microscopic studies were done using light microscopy, Confocal Laser Scanning Microscopy (CLSM), and gene expression studies for twitching motilities to compare strong and weak biofilm producers. In addition, biofilm composition (eDNA, extracellular protein, exopolysaccharides, pyocyanin, and rhamnolipids) were also compared. Through crystal violet assay results it was observed

that n=16 were strong, n=2 were moderate, and n=4 were weak biofilm producers. Majority of the isolates produced strong biofilm formation. The biofilm formation was high in presence of LB followed by natural urine (NU), and artificial urine (AU) of studies on catheters (silicone-coated latex and silicone catheter). A difference was found between strong and weak biofilm producers on silicone catheters in presence of LB and a negligible difference was observed in other conditions. Further, adhesion ability, twitching motility, dead and total (live and dead) number of cells were found to be higher in strong biofilm producers. Apart from this eDNA, an extracellular protein and pel polysaccharide was found to be high in strong biofilm producers. The high adhesion ability and twitching motility would cause an increase in biofilm formation by strong biofilm producers. As well as dead cells along with the total number of cells that would contribute to biofilm formed by strong biofilm producers.

One of the biofilm-mediated resistance mechanisms is PC formation was studied through stringent response genes (*relA*, *spoT,* and *lon*) and Toxin-antitoxin (TA) (*higB,* and *higA*) system genes through gene expression analysis in planktonic as well as biofilm stage at supra-MIC level (here 5X MIC) of antibiotic treatments. Study was carried out using three different antibiotics (ceftazidime, gentamicin, and ciprofloxacin) based on the mode of action. Initially, PC formation was studied through time kill assay at above MIC levels, flow cytometry (FC) for planktonic cells, and CLSM for biofilm using RSG and PI staining, and RT-PCR were carried out. First, the Minimum Inhibitory Concentration (MIC) of TP-10 showed susceptibility to all antibiotics while ST-13 was a resistant isolate and PAO1 was susceptible to two antibiotics (ceftazidime and gentamicin) and resistant to ciprofloxacin in planktonic stage ceftazidime showed least killing followed by gentamicin and ciprofloxacin which varied across the isolates. Quantitative data of RSG-positive cells through FC analysis showed the redox active cells on antibiotic treatment varied across the isolates. The RSG-stained cells after treatment with 5X MIC were visualized by fluorescence microscopy. PAO1 and TP-10 on ceftazidime treatment had elongated cells having high redox active cells. While gentamicin treatment resulted in rod-shaped cells with redox-active cells. And ciprofloxacin treatment had a smaller number of redox-active cells and dead cells were high in number. The transcriptional levels of stringent response varied across the isolates on different 5X MIC levels of antibiotic treatments. While transcription of *higB* and *higA* was observed on ceftazidime treatment and gentamicin treatment in PAO1 and TP-10 isolate and no expression was observed on ciprofloxacin treatment. While in ST-13 no difference in *higB* and *higA* was observed on the antibiotic treatments. PC formation was also studied in the biofilm stage. Biofilm quantification

and categorization showed PAO1 and ST-13 were strong biofilm producers while TP-10 was weak biofilm producers. Biofilm kill curve assay was done for all biofilm formed by three isolates. On Ceftazidime treatment the biofilm was regrown as compared to growth control (no antibiotic was added). While gentamicin and ciprofloxacin treatment resulted in a kill curve that varied across the isolates due to variations in biofilm cells/ biofilm thickness. Next, FC showed ceftazidime treatment had high redox activity of cells in PAO1 and TP-10 followed by gentamicin and ciprofloxacin which varied across the isolates. Quantitative data of RSG-positive cells showed a negligible difference in all biofilm formed by isolates on antibiotic treatments. Further, CLSM was done where untreated showed variation in thickness among the biofilm formed by isolates. On ceftazidime treatment, elongated cells were observed in PAO1 and TP-10 biofilm having redox active cells. While in ST-13, cell elongation was less and redox-active cells were also less in number. On gentamicin treatment, rod-shaped cells were observed to have redox active cells high in number in PAO1, TP-10, and ST-13 biofilms. On ciprofloxacin treatment, the biofilm was disrupted and dead cells were observed among the biofilm formed by isolates. The redox-active cells were also smaller in number. Gene expression was carried out after 5X MIC antibiotic treatment and normalized to untreated control. The gene expression of stringent response (*relA*, *spoT*, and *lon*) genes differed across the biofilm formed by isolates.

Another perspective of this study was to study biofilm resistance through Biofilm Resistance Locus Regulator (brlR) regulated by Surface attachment and growth sensor hybrid SagS The activated *brlR* in turn activates the multidrug efflux pumps (*mexAB-oprM*, *mexEF-oprN*). However, the role of negative regulators on biofilm resistance in not known. The biofilm resistance through specific gene expression was studied using whole genome sequencing (WGS), biofilm quantification, biofilm susceptibility assay, gene expression of *sagS, brlR, mexA, mexF, nalC* and *nalD* expression. For this study TP-10, TP-11 and PAO1 isolates were used for this studies. Through whole genome sequence analysis it was observed that there were transitional and transversional point mutation either leading to silent or substitution mutations observed in TP-10 (*brlR, mexA, mexB, nalC, mexE, nalD*) and TP-11 (*sagS*, *brlR, mexA, mexB, nalC,* and *mexE*) isolate. While in TP-10 isolate *sagS* gene was truncated and in TP-11 isolate nalD gene was 2 bp deleted. Further, through MIC study it was observed that TP-10 isolate was susceptible to gentamicin and ciprofloxacin antibiotics, TP-10 was resistant to both antibiotics whereas POA1 was susceptible to gentamicin and resistant to ciprofloxacin. Further, TP-10, and TP-11 were weak biofilm producer, while PAO1 was strong biofilm producers. No

significance log cfu/ml reduction was observed on biofilm susceptibility against gentamicin and ciprofloxacin antibiotics. Additionally, significance difference was observed in pycyanin production only in TP-11 isolate after treatment with anbiotics. On gentamicin and ciprofloxacin treatment the gene expression of *sagS, brlR, mexA, mexF* were upregulated in TP-11 isolate compared to TP-10 (Δ*sagS*). From the above study it can be said that there more biofilm resistance in TP-11 due to expression of *sagS, brlR, mexA, mexF* on both antibiotic treatments and GC.