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Association of exopolysaccharide genes in biofilm developing antibiotic-resistant *Pseudomonas aeruginosa* from hospital wastewater

M. Divyashree [a,*], Madhu K. Mani [a] and Indrani Karunasagar [b]

[a] Department of Biomedical Sciences, Nitte University Center for Science Education and Research (NUCSER), NITTE (Deemed to be University), Paneer Campus, Kotekar-Beeri Road, Deralakatte, Mangalore 575018, India
[b] DST-TEC, NITTE (DU), Paneer Campus, Kotekar-Beeri Road, Deralakatte, Mangaluru 575018, Karnataka, India
*Corresponding author. E-mail: divyashree.m@nitte.edu.in

MD, 0000-0002-5545-0696; MKM, 0000-0002-4368-4911

ABSTRACT

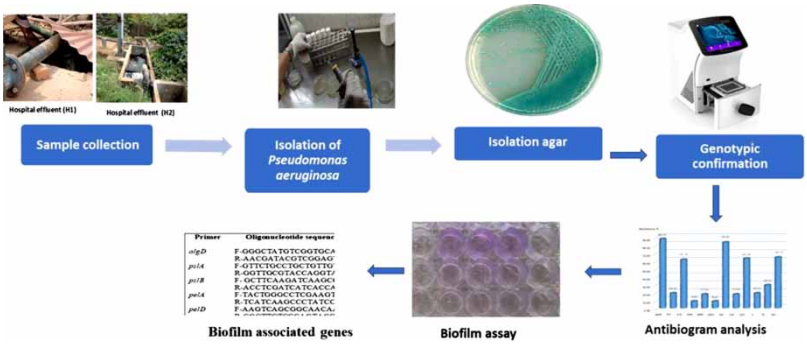
The study aimed to examine the relationship between antibiotic resistance, biofilm formation and genes responsible for biofilm formation. Sixty-six *Pseudomonas aeruginosa* isolates were obtained from hospital wastewater and analyzed for their antibiotic resistance. Biofilm production among the isolates was tested by indirect quantification method crystal violet assay. Biofilm-associated genes among these isolates *psl, alg*, and *pel* were also checked. The maximum resistance was observed for ampicillins (88.24%) followed by nalidixic (83.82%), and nitrofurantoin (64.71%), respectively. Biofilm phenotypes are distributed in the following categories: high 39.39% ($n = 26$); moderate 57.57% ($n = 38$), and weak 3.0% ($n = 2$). Among the total isolates, biofilm-associated genes were detected in 84.84% ($n = 56$) of isolates and the remaining isolates 15.15% ($n = 10$) did not harbor any genes. In this study, *pslB* was the most predominant gene observed (71.21%, $n = 47$) followed by *pslA* (57.57%, $n = 38$), *pelA* (45.45%, $n = 30$), *algD* (43.93%, $n = 29$), and *pelD* (27.27%, $n = 18$), respectively. The present study reveals that the majority of the isolates are multidrug resistant being moderate and high biofilm formers. The study implies that biofilm acts as a machinery for bacteria to survive in the hospital effluent which is an antibiotic stress environment.

Key words: antibiotic resistance, biofilm, exopolysaccharides, hospital, *Pseudomonas aeruginosa*, wastewater

HIGHLIGHTS

- Hospital effluent carrying biofilm forming antibiotic resistant bacteria is a major threat to public health.
- Dissemination of these bacteria into the surrounding environment is of major concern.
- Bacterial biofilm formation enhances the antibiotic resistance.
- Infection caused by these multidrug resistant isolates are difficult to treat.
- Study emphasizes the need of appropriate wastewater treatment.

GRAPHICAL ABSTRACT



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INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous, Gram-negative human opportunistic pathogen associated with several human infections leading to high morbidity, and mortality in healthcare settings and the community. *Pseudomonas* is one of the widely used model organisms to study bacterial biofilms (Ghafoor *et al.* 2011). Biofilm is an association of microorganisms which adhere to surfaces by self-producing a matrix of extracellular polymeric substances (Jamal *et al.* 2018). Bacterial biofilms are usually formed during the infection, where bacteria switch from a planktonic mode of growth to pathogenic form by forming an encapsulated matrix (Baker *et al.* 2015). Biofilms show resistance to the human immune system as well as resistance to antibiotic action including third- and fourth-generation cephalosporins and carbapenems (Lima *et al.* 2018).

Biofilm formation involves several steps starting with attachment to a living or non-living surface that will lead to formation of a micro-colony, giving rise to three-dimensional structures and ending up, after maturation, with detachment (Jamal *et al.* 2018). Biofilm composition varies but, generally, is composed of exopolysaccharides, nucleic acids, and proteinaceous adhesins and plays a functional role in biofilm formation. These exopolysaccharides can serve as a structural and protective role in antibiotic defense, biofilm matrix, and provides a barrier against phagocytosis of the human immune system (Mishra *et al.* 2012; Billings *et al.* 2013). Among different exopolysaccharides alginate, *Psl* and *Pel* are known to be produced by *P. aeruginosa* and are functionally important (Ryder *et al.* 2007; Ma *et al.* 2012; Yang *et al.* 2012). The role of *Pel* and *Psl* in biofilm formation may vary according to the strain; collectively, the studies have revealed that they function as structural scaffolds in maintaining the integrity of biofilms (Colvin *et al.* 2012). Phenotypic conversion of non-mucoid to mucoid colonies during cystic fibrosis by *Pseudomonas* spp. is associated with alginate production (Baker *et al.* 2015). Within a biofilm, bacteria communicate with each other by production of chemotactic particles or pheromones, a phenomenon called quorum sensing (Kleerebezem *et al.* 1997). Biofilm formation presents a challenge for clinicians in treating infection, since they colonize in catheters and ventilator tubes causing respiratory infection, particularly ventilator-associated pneumonia (VAP). Biofilm on biomaterials, within hospital surfaces, and water supplies pose a host of threats to vulnerable patients (Mulcahy *et al.* 2014).

Pseudomonas spp. exhibit intrinsic resistance to many antimicrobials under selective pressure. Thus, they easily develop powerful resistance either by chromosomal mutation or due to horizontal transfer of resistance genes (Zhao & Hu 2010). The infections caused by the multidrug resistance pathogen are often difficult to treat (De Francesco *et al.* 2013). *Pseudomonas* isolates show remarkable ability to adapt and thrive in different environmental conditions including soil, water, municipal wastes, clinical settings, and hospital wastes (Igbinosa *et al.* 2014; Igbinosa *et al.* 2017).

Pseudomonas spp. has a higher prevalence in hospital settings, hospital wastes, and their resistance is increasingly being recognized as a serious threat (Imanah *et al.* 2017; Divyashree *et al.* 2020). Several studies have reported the presence of antibiotic-resistant *Pseudomonas* spp. from human and non-human origins. The present study aimed to appraise the relationship between antibiotic resistance phenotype, as well as phenotypic and genotypic characteristics of the biofilm in *P. aeruginosa*.

MATERIALS AND METHODS

Sample collection

Effluent samples ($n = 55$) were collected from the untreated wastewater outlet pipe of two tertiary care hospitals (H1 and H2) in and around Mangalore, India (before their entry into the wastewater treatment plant (WWTP)). Effluent samples were collected in 100 mL sterile bottles from the respective sources and transported to the laboratory for microbiological analysis.

Isolation of *Pseudomonas aeruginosa*

To obtain the isolated colonies, serial dilution (10^{-1}, 10^{-2}, and 10^{-3}) of the samples with sterile physiological saline was prepared. One hundred μL of each aliquot of serial ten-fold dilutions made in saline were spread plated on selective media *Pseudomonas* isolation agar, and incubated at a temperature of 37 °C for 24 h. The colonies were subjected to a series of biochemical tests for identification.

Molecular confirmation of *Pseudomonas* spp.

Genotypic confirmation of *Pseudomonas spp*. was done by polymerase chain reaction (PCR) using specific primers (OprL1 F: ATG GAA ATG CTG AAA TTC GGCCT; OprL2 R: T CTT CAG CTC GAC GCG ACG, annealing temperature 55 °C, amplicon size 504 bp). All identified isolates were preserved in Luria Bertani (LB) broth with 30% glycerol and stored at −80 °C.

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Antibiotic susceptibility testing

Antibiotic susceptibility test was performed by Kirby–Bauer disk diffusion method using ATCC strains as control. Isolates were classified as sensitive, intermediate, and resistant based on the inhibition zone diameter around the antibiotic disks, according to Clinical and Laboratory Standards Institute guidelines. The following antimicrobial disks (Oxoid) were used: ampicillin (AMP); piperacillin-tazobactam combination (PIT); cefotaxime (CTX); ceftazidime (CAZ); imipenem (IMP); meropenem (MRP); gentamicin (GEN); nalidixic acid (NA); ciprofloxacin (CIP); cotrimoxazole (COT); chloramphenicol (C); tetracycline (TE); and nitrofurantoin (NIT). The organisms were grown in Mueller-Hinton (MH) (HiMedia, India) broth for 4–6 h, the turbidity was adjusted to 0.5 McFarland's standard and was spread using sterile cotton swabs on well-dried Mueller-Hinton Agar (MHA) (HiMedia, India) plates to prepare a lawn. After gently air drying in a laminar flow chamber, the respective antibiotic disks were placed on the surface of the plates and incubated for 24 h at 37 °C for the appearance of clear zone. The diameter of clear zones was taken and interpreted as sensitive and resistance according to the CLSI guidelines using ATCC strains *Pseudomonas aeruginosa* PAO1 as control. Isolates were considered as multidrug-resistant (MDR) if they showed resistance to three or more classes of the tested antibiotics and resistance (R) for one or two antibiotics.

Quantification of biofilm

Biofilm assay

The biofilm quantification was performed using a previously described microtiter dish biofilm formation assay method (O'Toole 2011) with small modifications. The isolates of *P. aeruginosa* were cultured in LB broth for 24 h at 37 °C. *P. aeruginosa* PAO1 (biofilm producing) and *P. aeruginosa* ATCC 27853 (non-biofilm producing) were used as controls. For microtitration, 200 μL of the bacterial suspensions were added to the polystyrene plates containing 96 flat-bottom wells in triplicate. The LB broth with the ATCC culture 27853 was used as the negative control, and *P. aeruginosa* strain PAO1 was used as the positive control since this strain is recommended as a positive control for biofilm assays. The plates were then incubated at 37 °C for 72 h and the results for biofilm production were taken at time intervals of 24, 48, and 72 h. The experiments were done in triplicate. The bacterial suspensions were then removed, and each well was washed three times with PBS (pH 4.0). Subsequently, the plates were stained with 225 μL of crystal violet (CV-0.1%) solution for 10 min. The plates were then washed with PBS, added to 30% glacial acetic acid, and incubated at room temperature for 15 min. After this process, 100 μL of the solubilized CV were transferred to a flat-bottomed microtiter dish and absorbance readings were taken in an ELISA reader (BioTek, ELx800) at wavelength of 550 nm, and the samples were classified as non-biofilm, weak, moderate, and high biofilm formers ($OD \leq 0.071$ – non-biofilm; $OD \leq 0.071$–0.142 weak; $OD \leq 0.142$–0.284 moderate; $OD \leq 0.284$ high) (Perez *et al.* 2011).

Bacterial crude DNA extraction

One mL of 24 h-grown culture in LB broth was taken in a 1.5 mL microcentrifuge tube and culture was centrifuged at 5,000 rpm for 10 min. The supernatant was discarded and the pellet was dissolved in 300 μL of 1XTE buffer. The tubes were placed in a dry bath at 95 °C for 10 min and then immediately placed in ice for another 10 min. After centrifugation at 5,000 rpm for 5 min the supernatant was transferred to a new tube and the crude DNA was preserved at −20 °C for further use. The DNA isolated from *P. aeruginosa* cultures was subjected to PCR to detect the biofilm-associated genes using specific primers listed in Table 1.

Table 1 | Primers and primer sequence used for identification of biofilm-associated genes

Primer	Oligonucleotide sequence (5′–3′)	Amplicon size (bp)
algD	F-GGGCTATGTCGGTGCAGTAT; R-AACGATACGTCGGAGTCCAG	219
pslA	F-GTTCTGCCTGCTGTTGTTCA; R-GGTTGCGTACCAGGTATTCG	230
pslB	F-GCTTCAAGATCAAGCGCATC; R-ACCTCGATCATCACCAGGTC	220
pelA	F-TACTGGGCCTCGAAGTTCTC; R-TCATCAAGCCCTATCCGTTC	214
pelD	F-AAGTCAGCGGCAACAACAC; R-CGCTTCTCCCAGTACCTCAA	208

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Polymerase chain reaction (PCR)

PCR was carried out in 30 μL reaction mixture containing 10X buffer (100 mM Tris-HCl, pH 8.3, 20 mM $MgCl_2$, 500 mM KCl, 0.1% gelatin), 200 mM of dNTPs, 10 pmol each of forward and reverse primers, and 1.0 unit of *Taq* DNA polymerase enzyme (Bangalore Genie, Bangalore). In a sterile PCR tube, 22.1 μL of sterile distilled water, 3 μL of 10X assay buffer, 0.6 μL of 200 mM dNTP mix, 2 μL of each primer (forward and reverse) (10 pmol/μL), 0.3 μL of *Taq* polymerase, and 2 μL of template DNA solution was taken. Amplification was carried out in a MJ-Research Thermo Cycler (PTC-200, USA) with the optimized PCR program that consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles. Each cycle comprised denaturation at 94 °C for 60 sec, temperature of 55 °C for 60 sec, and extension at 72 °C for 30 sec. The final extension included one cycle at 72 °C for 10 min. The amplicons were resolved by electrophoresis in 1.5% agarose gel, stained with ethidium bromide and visualized in a gel documentation system (Bio-Rad, USA).

RESULTS

Out of 55 effluent samples, 66 isolates were confirmed as *P. aeruginosa* using a battery of biochemical tests and molecular methods. The antibiotic resistance and the ability of biofilm formation of all 66 isolates of *P. aeruginosa* were examined. The isolates showed resistance to most of the commonly used antibiotics tested. Of 66 *Pseudomonas* isolates, 59 (89.39%) were multidrug-resistant (resistant to three or more classes of antibiotics), and the remaining seven isolates showed resistance to one or two different classes of antibiotics. Isolates showed maximum resistance to ampicillin (88.24%) and nalidixic (83.82%), respectively. The resistance to other antibiotics is presented in Figure 1.

Biofilm phenotypes of the isolates were studied by quantitative assay method. Among the total isolates ($n = 66$), 39.39% ($n = 26$) of isolates were found to be high biofilm producers (OD = >0.284) at 24-h time interval. Moderate biofilm formation was observed among 57.57% of the isolates ($n = 38$). Two isolates (3.0%, $n = 2$), namely, 327 K and 384Y were found to be weak biofilm (Table 2). Biofilm forming ability of the isolates were checked using positive and negative controls (high OD >0.284, moderate 0.142–0.284, weak 0.071–0.142 at 600 nm 24-h time point).

All 66 *P. aeruginosa* were tested for the biofilm forming genes. Among them, 56 (84.84%) of isolates harbored at least one of the biofilm genes tested. The high occurrence of gene *pslB* was observed in 71.21% ($n = 47$) of isolates followed by *pslA* (57.57%, $n = 38$), *pelA* (45.45%, $n = 30$), *AlgD* (43.93%, $n = 29$), and *pelD* (27.27%, $n = 18$) isolates, respectively. In this study, 10.60% ($n = 7$) of isolates harbored all the genes ($algD + Psl\ A + Psl\ B + Pel\ A + Pel\ D +$ genotypic pattern) tested and their biofilm capacity ranged from moderate to high, while 15.15% ($n = 10$) of isolates did not harbor any of the biofilm genes ($algD^-$, $Psl\ A^-$, $Psl\ B^-$, $Pel\ A^-$, $Pel\ D^-$ genotypic pattern). Among these ten isolates, two showed high biofilm

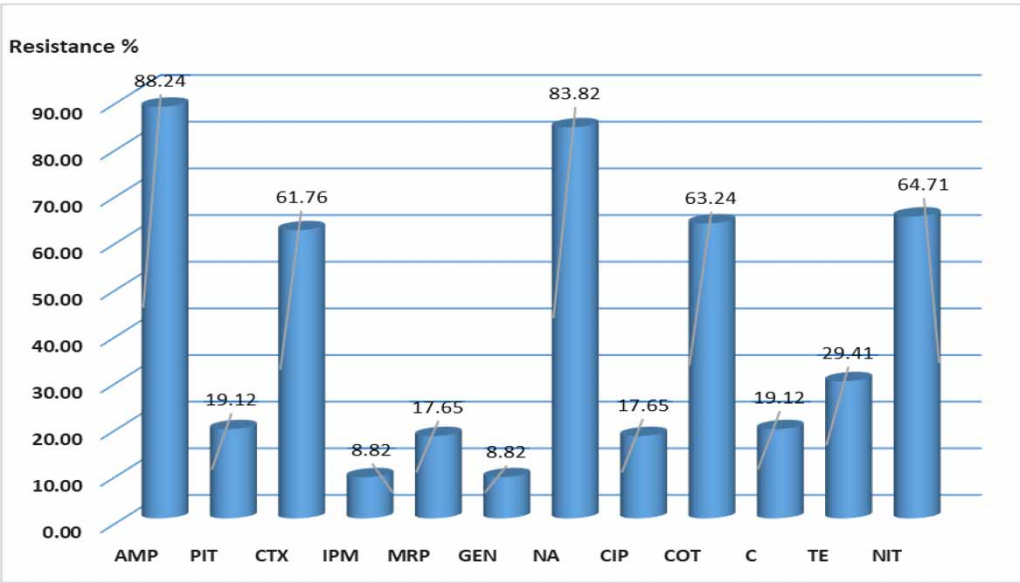

Figure 1 | Antibiotic resistance pattern of *Pseudomonas aeruginosa* isolates.

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Table 2 | *Pseudomonas aeruginosa* isolates showing antibiotic resistance phenotype, biofilm phenotype and genotype

		Biofilm-associated genes					
No.	**Resistance phenotype**	***algD***	***Psl A***	***Psl B***	***Pel A***	***Pel D***	**Biofilm formation at 24 hrs**
42 K	MDR	−	−	+	−	−	High
43 K	R	−	−	+	−	−	High
56 K	MDR	+	+	+	+	+	High
57 K	MDR	+	+	+	+	+	Moderate
58 K	MDR	+	+	+	+	+	High
59 K	MDR	+	+	+	+	+	Moderate
60 K	MDR	+	+	+	+	−	High
61 K	MDR	+	+	+	+	−	High
62 K	MDR	+	+	+	+	−	High
63 K	MDR	+	+	+	+	−	High
64 K	MDR	+	+	+	+	−	Moderate
65 K	MDR	−	+	+	+	−	Moderate
66 K	MDR	+	−	+	+	−	High
67 K	MDR	+	+	+	+	−	High
68 K	MDR	+	+	+	+	−	Moderate
69 K	R	−	+	+	+	−	Moderate
70 K	MDR	+	+	+	+	−	High
71 K	MDR	+	+	+	+	−	Moderate
72 K	MDR	−	+	+	−	−	Moderate
73 K	MDR	+	+	+	+	−	Moderate
74 K	MDR	+	+	+	−	−	Moderate
75 K	MDR	−	−	+	−	−	Moderate
326 K	MDR	−	+	+	−	−	Moderate
327 K	MDR	+	−	−	+	−	Weak
328 K	MDR	+	+	+	+	+	High
329 K	MDR	+	+	+	+	+	Moderate
374 K	MDR	−	+	−	−	−	Moderate
375 K	MDR	+	+	+	+	+	Moderate
376 K	MDR	−	+	+	+	+	Moderate
377 K	MDR	−	−	+	+	+	Moderate
378 K	MDR	−	−	+	−	−	High
379 K	MDR	−	−	−	−	−	Moderate
380 K	MDR	−	−	+	+	+	Moderate
381 K	MDR	−	−	−	−	−	Moderate
382 K	MDR	−	−	−	−	−	Moderate
383 K	MDR	−	−	−	+	+	Moderate
150Y	MDR	−	+	−	−	−	High
151Y	R	+	+	+	−	+	High
152Y	MDR	+	+	−	+	+	High
153Y	MDR	−	+	+	−	−	High
154Y	MDR	+	+	+	−	−	High

(*Continued.*)

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Table 2 | Continued

No.	Resistance phenotype	Biofilm-associated genes					Biofilm formation at 24 hrs
		algD	*Psl A*	*Psl B*	*Pel A*	*Pel D*	
155Y	R	−	−	+	−	−	Moderate
188Y	MDR	−	−	−	−	−	Moderate
189Y	MDR	−	−	−	−	−	High
191Y	MDR	−	−	−	−	−	High
230Y	MDR	+	+	+	−	+	High
231Y	MDR	−	+	+	+	−	Moderate
232Y	MDR	+	+	+	−	+	High
233Y	MDR	+	+	+	+	−	Moderate
234Y	MDR	−	−	−	+	−	Moderate
235Y	MDR	+	+	+	−	−	Moderate
236Y	MDR	−	+	−	−	−	Moderate
240Y	MDR	−	+	+	−	−	Moderate
241Y	MDR	−	+	+	−	−	High
242Y	MDR	+	+	+	−	+	Moderate
280Y	MDR	−	−	+	−	−	Moderate
281Y	MDR	−	−	+	−	−	High
282Y	MDR	−	−	+	−	−	High
283Y	MDR	−	−	+	−	−	High
286Y	MDR	−	−	+	−	−	Moderate
371Y	R	−	−	−	+	+	Moderate
372Y	MDR	−	−	−	−	−	Moderate
373Y	R	−	−	−	−	−	Moderate
384Y	R	−	−	−	−	−	weak
385Y	MDR	−	−	−	−	−	Moderate
386Y	MDR	+	−	−	−	+	Moderate

producers, seven moderate, and one weak. However, it is interesting to note that the weak biofilm formers in the present study (327 K, 384Y) showed either the presence or absence of genes. The isolate 327 K harbored *algD* and *PelA* while the isolate 384Y did not harbor any biofilm-associated genes.

In addition, the isolates were divided into two groups based on their antibiotic resistance pattern (MDR, R) and their biofilm characteristics (Figure 2). Among the 59 MDR isolates 34 (57.62%) are moderate biofilm formers, 24 (40.67%) high, and 1 (1.69%) weak, while of the resistance isolates ($n = 7$), four showed moderate, two high, and one weak biofilm formers (Figure 2).

DISCUSSION

The presence of antibiotic-resistant *P. aeruginosa* in hospital wastewater, surface water, and other environmental samples constitutes an important public health threat (Fuentefria *et al.* 2010; Divyashree *et al.* 2020). Antibiotic treatment fails to eradicate the biofilms due to their intrinsic antibiotic resistance and the development of mutational antibiotic resistance. The resistance of the biofilm to antibiotics involves many factors such as physiological, physical, genotypic resistance and repeated exposure of the biofilm forming bacteria to antibiotics (Ciofu & Tolker-Nielsen 2019).

The high prevalence of multidrug resistance *P. aeruginosa* in the present study (89.39%) is an alarming situation which mirrors the threat limiting treatment option in these hospital settings. The study conducted by Bavasheh & Karmostaji

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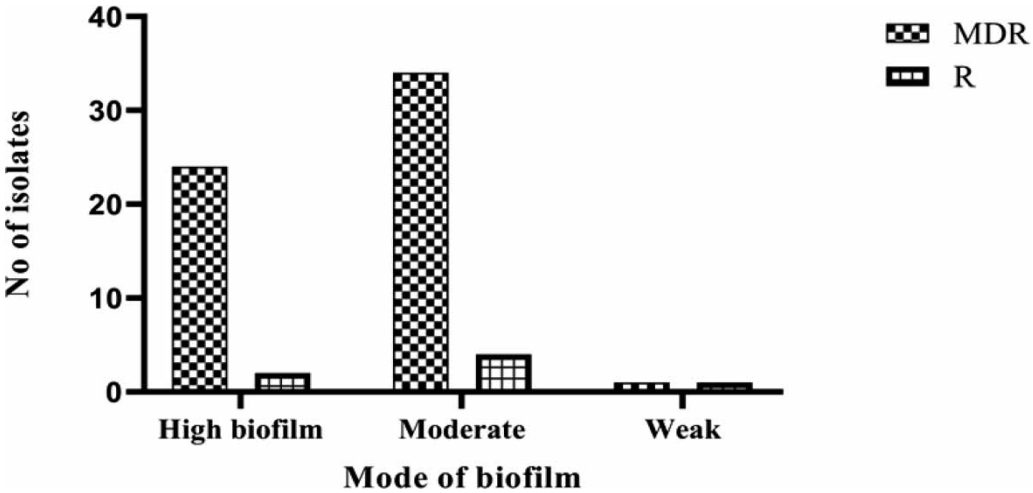

Figure 2 | Relationship between antibiotic resistance and biofilm characteristic and among *P. aeruginosa* clinical isolates.

(2017) reported 27.8% of clinical *P. aeruginosa* isolates were MDR. The isolates in the study exhibited high resistance against ampicillin (88.24%) and nalidixic (83.826%), respectively. In another study, clinical isolates of *P. aeruginosa* showed high resistance towards meropenem (30.6%) and ticarcillin (22.3%), followed by other antibiotics (Khan & Faiz 2016). Of 59 MDR isolates, 24 (40.67%) were high biofilm formers. The present study supports another study in Brazil in which 48.4% were biofilm formers (Lima *et al.* 2018). Biofilm production was significantly higher in MDR isolates reported by Abidi *et al.* (2013).

P. aeruginosa biosynthesizes the exopolysaccharide which is a key component for colonization, biofilm formation, and provides protection to the opportunistic pathogen (Jackson *et al.* 2004). Three important distinct exopolysaccharides involved in biofilm formation includes *Psl*, *Alg*, and *Pel* (Franklin *et al.* 2011). The present study revealed the high prevalence of *pslB* (71.21%) followed by *pslA* (57.57%), *AlgD* (43.93%), *pel A* (45.45%), and *pel D* (27.27%), respectively, being presented among *P. aeruginosa* isolates. In contrast to this, the study by Kamali *et al.* (2020) reported the prevalence of *algD*, *pslD*, and *pelF* genes among 87.5% of *P. aeruginosa* isolates. The genes related with biofilm formation *pslA* and *pelA* with a frequency of 83.7% and 45.2%, respectively, was reported by Ghadaksaz *et al.* (2015). Although the present study does not state directly that *psl* genes are novel exopolysaccharides for high biofilm formation, because the isolates carrying these genes showed variation in biofilm formation from moderate to high. According to Hou *et al.* (2012), the inability of the *P. aeruginosa* isolates to show phenotypic biofilm formation but being positive for *pslA* gene could be due to mutation in the quorum sensing proteins, which was not studied in the present study.

Alginate (Alg gene) is the predominant extracellular polysaccharide in mucoid strains frequently isolated from the lungs of chronically colonized cystic fibrosis patients (Colvin *et al.* 2012). About 43.93% of *P. aeruginosa* isolates in this study carried the *algD* gene, but the study by Ghadaksaz *et al.* (2015) reported high *algD* (87.5%) prevalence. It is important to know from the current study that apart from two isolates, 189Y and 191Y, all other high biofilm forming isolates harbored at least one of the *psl* gene (*pslA* or *pslB*) tested.

CONCLUSION

According to the present study results, *P. aeruginosa* that are high, moderate, and weak biofilm formers and the associated genes have some relationship, because one isolate which was a weak biofilm former does not harbor any biofilm-associated gene while the other isolate carried two genes, *algD* and *PelA*, respectively. Thus, we can assume that the gene *psl* is important for the isolate to form biofilm and their antibiotic resistance phenotype does not have any relationship with biofilm formation. The reasons for the biofilm formation between MDR and resistance isolates and the genes associated with biofilm formation is likely multifactorial. Hence, studies on expression of these genes and other virulence factors may help to draw a conclusion.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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