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

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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 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 GGCCCT; 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 50% glycerol and stored at −80 °C.
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/C20.071–0.142 weak; OD/C20.142–0.284 moderate; OD/C0.284 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-GGGCTATATGTCGGTGACGAT; R-AAAGATATACGTCGGAGTCCAG | 219 |
| pslA   | F-GTTCGCTGCTGTGTTGTTCA; R-GTTCGCGTACAGGTATTCG | 230 |
| pslB   | F-GCTTCAGATGATCAGGGCATC; R-ACCTGATCATCACCAGGTC | 220 |
| pelA   | F-CTACGGGCTCAGATTTC; R-TATACAGCCCTATCAG | 214 |
| pelD   | F-AACGTCAGCGCAACACAC; R-CGCTTCTCCAGTACCTCAA | 208 |
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 MgCl2, 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 formation.
Table 2 | *Pseudomonas aeruginosa* isolates showing antibiotic resistance phenotype, biofilm phenotype and genotype

| No.  | Resistance phenotype | Biofilm-associated genes | Biofilm formation at 24 hrs |
|------|---------------------|--------------------------|-----------------------------|
|      |                     | algD | PsI A | PsI B | PeI A | PeI D |                      |
| 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              |
| 76 K | MDR                 | –    | –     | +     | –     | –     | Moderate              |
| 77 K | MDR                 | –    | –     | +     | +     | +     | Moderate              |
| 78 K | MDR                 | –    | –     | +     | –     | –     | High                  |
| 79 K | MDR                 | –    | –     | +     | –     | –     | Moderate              |
| 80 K | MDR                 | –    | –     | +     | +     | +     | Moderate              |
| 81 K | MDR                 | –    | –     | –     | –     | –     | Moderate              |
| 82 K | MDR                 | –    | –     | –     | –     | –     | Moderate              |
| 83 K | MDR                 | –    | –     | –     | –     | –     | Moderate              |
| 150Y| MDR                 | –    | +     | –     | –     | –     | High                  |
| 151Y| R                   | +    | +     | +     | –     | +     | High                  |
| 152Y| MDR                 | +    | +     | –     | +     | +     | High                  |
| 153Y| MDR                 | –    | +     | +     | –     | –     | High                  |
| 154Y| MDR                 | +    | +     | +     | –     | –     | High                  |

(Continued.)
producers, seven moderate, and one weak. However, it is interesting to note that the weak biofilm formers in the present study 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 (Fuenteferia 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
(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 include *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.

REFERENCES

Abidi, S. H., Sherwani, S. K., Siddiqui, T. R., Bashir, A. & Kazmi, S. U. 2013 Drug resistance profile and biofilm forming potential of Pseudomonas aeruginosa isolated from contact lenses in Karachi-Pakistan. BMC Ophthalmology 13, 57.

Baker, P., Whitfield, G. B., Hill, P. J., Little, D. J., Pestruk, M. J., Robinson, H., Wozniak, D. J. & Howell, P. L. 2015 Characterization of the Pseudomonas aeruginosa glycose hydrolase PsIG reveals that its levels are critical for PsI polysaccharide biosynthesis and biofilm formation. Journal of Biological Chemistry 290 (47), 28574–28587.

Bavasheh, N. & Karmostaji, A. 2017 Antibiotic resistance pattern and evaluation of blaOXA-10, blaPER-1, blaVEB, blaSHV genes in clinical isolates of Pseudomonas aeruginosa isolated in south of Iran in 2014–2015. Infection Epidemiology and Medicine 3 (1), 1–5.

Billings, N., Millan, M., Caldara, M., Rusconi, R., Tarasova, Y., Stocker, R. & Ribbeck, K. 2013 The extracellular matrix component PsI provides fast-acting antibiotic defense in Pseudomonas aeruginosa biofilms. PLoS Pathogens 9, e1003526.

Ciofu, O. & Tolker-Nielsen, T. 2019 Tolerance and resistance of Pseudomonas aeruginosa biofilms to antimicrobial agents – How P. aeruginosa can escape antibiotics. Frontiers in Microbiology 10, 913.

Colvin, K. M., Irie, Y., Tart, C. S., Urbano, R., Whitney, J. C., Ryder, C., Howell, P. L., Wozniak, D. J. & Parsek, M. R. 2012 The Pel and Psl polysaccharides provide Pseudomonas aeruginosa structural redundancy within the biofilm matrix. Environmental Microbiology 14 (8), 1913–1928.

De Francesco, M. A., Ravizzola, G., Peroni, L., Bonfanti, C. & Manca, N. 2013 Prevalence of multidrug-resistant Acinetobacter baumannii and Pseudomonas aeruginosa in an Italian hospital. Journal of Infection and Public Health 6 (3), 179–185.

Divyashree, M., Mani, K. M., Shama, P. K., Vijaya, K. D., Veena, S. A., Shetty, A. K. & Karunasaragit, I. 2020 Hospital wastewater treatment reduces NDM-positive bacteria being discharged into water bodies. Water Environment Research 92 (4), 562–568.

Franklin, M. J., Nivens, D. E., Weadge, J. T. & Howell, P. L. 2011 Biosynthesis of the Pseudomonas aeruginosa extracellular polysaccharides, alginate, Pel, and PsI. Frontiers in Microbiology 2, 1–16.

Fuentesfria, D. B., Ferreira, A. E. & Coçraô, G. 2010 Antibiotic-resistant Pseudomonas aeruginosa from hospital wastewater and superficial water: are they genetically related? Journal of Environmental Management 92 (1), 250–255.

Ghadakza, A., Fooladi, A. A. I., Hosseini, H. M. & Amin, M. 2015 The prevalence of some Pseudomonas virulence genes related to biofilm formation and alginate production among clinical isolates. Journal of Applied Biomedicine 13, 61–68.

Ghafour, A., Hay, I. D. & Rehm, B. H. A. 2011 Role of exopolysaccharides in Pseudomonas aeruginosa biofilm formation and architecture. Applied Environmental Microbiology 77 (15), 5238–5246.

Hou, W., Sun, X., Wang, Z. & Zhang, Y. 2012 Biofilm-forming capacity of Staphylococcus epidermidis, Staphylococcus aureus, and Pseudomonas aeruginosa from ocular infections. Investigative Ophthalmology & Visual Science 53 (9), 5624–5631.

Igbinosa, I. H., Igbinosa, E. O. & Okoh, A. I. 2014 Molecular detection of metallo-β-lactamase and putative virulence genes in environmental isolates of Pseudomonas species. Polish Journal of Environmental Studies 25 (6), 2327–2331.

Igbinosa, I. H., Beshiru, A. & Igbinosa, E. O. 2017 Antibiotic resistance profile of Pseudomonas aeruginosa isolated from aquaculture and abattoir environments in urban communities. Asian Pacific Journal of Tropical Disease 7 (1), 47–52.

Imanah, E. O., Beshiru, A. & Igbinosa, E. O. 2017 Antibiogram profile of Pseudomonas aeruginosa isolated from some selected hospital environmental drains. Asian Pacific Journal of Tropical Disease 7 (10), 604–609.

Jackson, K. D., Starkey, M., Kremer, S., Parsek, M. R. & Wozniak, D. J. 2004 Identification of pel, a locus encoding a potential exopolysaccharide that is essential for Pseudomonas aeruginosa PA01 biofilm formation. Journal of Bacteriology 186 (14), 4466–4475.

Jamal, M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Nawaz, M. A., Hussain, T., Ali, M., Rafiq, M. & Kamil, M. A. 2018 Bacterial biofilm and associated infections. Journal of the Chinese Medical Association 81 (1), 7–11.

Kamali, E., Jamali, A., Ardebili, A., Ezadi, F. & Mohebbi, A. 2020 Evaluation of antimicrobial resistance, biofilm forming potential, and the presence of biofilm-related genes among clinical isolates of Pseudomonas aeruginosa. BMC Research Notes 13, 27.

Khan, M. A. & Faiz, A. 2016 Antimicrobial resistance patterns of Pseudomonas aeruginosa in tertiary care hospitals of Makkah and Jeddah. Annals of Saudi Medicine 36 (1), 23–28.

Kleerebezem, M., Quadri, L. E., Kuipers, O. P. & de Vos, W. M. 1997 Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Molecular Microbiology 24 (5), 895–904.

Lima, J. L. C., Alves, L. R., de Araújo Jácome, P. R. L., Neto, J. P. B., Maciel, M. A. & Morais, M. M. 2018 Biofilm production by clinical isolates of Pseudomonas aeruginosa and structural changes in LasR protein of isolates non biofilm-producing. The Brazilian Journal of Infectious Diseases 22 (2), 129–136.
Ma, L., Wang, S., Wang, D., Parsek, M. R. & Wozniak, D. J. 2012 The roles of biofilm matrix polysaccharide Psl in mucoid Pseudomonas aeruginosa biofilms. *FEMS Immunology & Medical Microbiology* 65 (2), 377–380.

Mishra, M., Byrd, M. S., Sergeant, S., Azad, A. K., Parsek, M. R., McPhail, L., Schlesinger, L. S. & Wozniak, D. J. 2012 Pseudomonas aeruginosa Psl polysaccharide reduces neutrophil phagocytosis and the oxidative response by limiting complement-mediated opsonization. *Cellular Microbiology* 14 (1), 95–106.

Mulcahy, L. R., Isabella, V. M. & Lewis, K. 2014 *Pseudomonas aeruginosa* biofilms in disease. *Microbial Ecology* 68 (1), 1–12.

O'Toole, G. A. 2011 Microtitre dish biofilm formation assay. *Journal of Visualized Experiments* 47, 2437.

Perez, L. R. R., Costa, M. C. N., Freitas, A. L. P. & Barth, A. L. 2011 Evaluation of biofilm production by *Pseudomonas aeruginosa* isolates recovered from cystic fibrosis and non-cystic fibrosis patients. *Brazilian Journal of Microbiology* 42 (2), 476–479.

Ryder, C., Byrd, M. & Wozniak, D. J. 2007 Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Current Opinion in Microbiology* 10 (6), 644–648.

Yang, L., Hengzhuang, W., Wu, H., Damkiaer, S., Jochumsen, N., Song, Z., Givskov, M., Høiby, N. & Molin, S. 2012 Polysaccharides serve as scaffold of biofilms formed by mucoid *Pseudomonas aeruginosa*. *FEMS Immunology & Medical Microbiology* 65 (2), 366–376.

Zhao, W. H. & Hu, Z. Q. 2010 Beta-lactamases identified in clinical isolates of *Pseudomonas aeruginosa*. *Critical Reviews in Microbiology* 36 (3), 245–258.

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