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# Assessment of biofilm development and identification of biofilm related genes in *Pseudomonas aeruginosa* strains isolated from mastitis infected Dorper milk

**Nor Azlinda Mohd Azhari Lutfi[1], Amirah Wan-Azemin[1], Nadiawati Alias*[1], Asmad Kari[2], Rosli Md Illias[3] and Arshad M. Ali[4].**

[1]School of Agriculture Science and Biotechnology, Faculty of Bioresources and Food Industry, UniversitiySultan Zainal Abidin, Besut Campus, 22200 Besut, Terengganu, **Malaysia**
[2]School of Animal Science, Aquatic Science and Environment, Faculty of Bioresources and Food Industry, University Sultan Zainal Abidin, Besut Campus, 22200 Besut, Terengganu, **Malaysia**
[3]School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, 81310 Johor Bahru, **Malaysia**
[4]Agropolitan Besut-Setiu, Panchur Bederu, Mukim Chalok, 21450 Setiu, Terengganu, **Malaysia**

***Correspondence:** nadiawati@unisza.edu.my

*Pseudomonas aeruginosa* is an opportunistic pathogen characterized by its biofilm formation ability and high-level multiple antibiotic resistance. The emergence of multidrug resistance (MDR) *Pseudomonas aeruginosa* has developed a great health challenges and affected global economy worldwide. Biofilm formation in *Pseudomonas aeruginosa* is controlled by its chromosomal genes (~1%). The aim of this study was to determine the biofilm formation in *P. aeruginosa* isolates and to screen the presence of biofilm related genes. Three strains were isolated from mastitis infected dorper milks from Agropolitan Farm Besut Setiu, Terengganu, Malaysia. Antibiotic susceptibility tests were performed and showed that *P. aeruginosa* ATCC BAA-2108 exhibit strong resistant against all the antibiotics group tested followed by *P. aeruginosa* ATCC 27853 and *P*. *aeruginosa* isolate 13(1) based on antibacterial disc diffusion assay. Other *P*. *aeruginosa* isolates 00(1) and 66(1) were found susceptible towards 11 antibiotics agents including carbapenems group (doripenem and meropenem). Quantification of biofilm formation of each strain was done by the crystal violet method using biofilm formation assay. According to the optical density (OD) readings, *P. aeruginosa* ATCC BAA-2108 exhibit strong adherent categories while other strains were classified into moderate and weak adherent. Screening for the selected biofilm genes (*pelA*, *pslA*, and *ndvB* genes) in each strain was performed using PCR, revealed the presence of these genes in each strain. These results suggested the importance of these genes in biofilm formation and the presence of other factors (genetic determinant and mutation) which may also contribute in determining the degree of biofilm formation in *P. aeruginosa*.

**Keywords: Biofilm formation assay, biofilm genes, *Pseudomonas aeruginosa*, mastitis, Dorper**

## INTRODUCTION

Biofilms are a cluster of microorganisms that attached to a surface and embedded in a matrix of extracellular polymeric substances (Cepas et al., 2019). The biofilm provide structure and protection against host's immune system and antimicrobial therapy (Okuda et al. 2018). The treatment and eradication of infection caused by biofilm-forming bacteria becomes a challenge and this suggests the need to develop new antimicrobials, which might also be a potential inhibitors of biofilm formation (Rasamiravaka et al.

2015). Many studies have been developed to elucidate the structural components of the biofilm matrix, the regulatory pathways involved in biofilm formation, and signaling molecules involved in biofilm formation which provide opportunities for prevention and control of these biofilms (Abee et al. 2011).

*Pseudomonas aeruginosa* is an opportunistic pathogen that are capable to cause acute and chronic infection and are well known to infect individuals with genetic disease such as cystic fibrosis (Colvin et al. 2012). *P. aeruginosa* has also been associated with mastitis in dairy animals (Kelly & Wilson, 2016). In a research conducted by Ezzeldeen et al. (2016), the prevalence of *P. aeruginosa* isolates were found 60% in goat milk, 41.2% in sheep milk, and 54.5% in raw cow milk in Ta'if region, Saudi Arabia (Ezzeldeen et al., 2016). *P. aeruginosa* are a model organism for biofilm studies due to its biofilm-forming abilities. *P. aeruginosa* growing in biofilm often show increased resistance to antibiotics compared to its planktonic state (Beaudoin et al. 2012).

Gene expression in biofilm and planktonic cells of *P. aeruginosa* differs about 1%, with about 0.5% of repressed genes and 0.5% activated genes (Sebak, 2013). Thus, it shows that biofilm formation is regulated by certain genes in *P. aeruginosa*. This strain produces three main exopolysaccharide in biofilm matrix; pellicle polysaccharide (pel), polysaccharide synthesis locus (psl) and alginate, which are regulated by the genes *pel*, *psl* and *alg* respectively (Marmont et al. 2017). *Pel* locus consist of seven genes (*pelA* to *pelG*) whereas the *psl* locus consist of 15 genes (*pslA* to *pslO*) (Friedman & Kolter, 2004; Ghafoor et al., 2011). *PelA* encodes a protein with a predicted polysaccharide deacetylase domain which interacts with *pelB* for the deacetylation of pel before it is exported from the cell (Colvin et al., 2012). Study by Ma et al. (2006) shows that disruption of *pslA* and *pslB* affect the biofilm initiation and attachment. Thus, it was predicted that both genes involve in the cell-surface and intercellular interactions to initiate biofilm formations (Emami et al., 2015; Ma et al., 2006). Another gene, *ndvB* was proved to be important for biofilm-specific resistance through a mechanism involving sequestration of the antibiotic by cyclic-β 1,3 glucans. It is a *ndvB*-derived glucan which is synthesized with glucosyltransferase encoded by *ndvB* gene (Zhang et al., 2013).

In this study, our aimed were to determine biofilm formation in *P. aeruginosa* isolates, their antibiotic susceptibility profile and also to screen the presence of biofilm related genes (*pelA*, *pslA* and *ndvB*) in the isolates.

## MATERIALS AND METHODS

### Sample Collection.

Milk samples were collected from lactating Dorper sheeps with mastitis from Agropolitan Farm Besut Setiu, Terengganu, Malaysia and the milk samples were tested for mastitis using California Mastitis Test (CMT) (Ariffin et al. 2019). Three *P. aeruginosa* strains were isolated and labelled as 13(1), 00(1) and 66(1). The isolated strains were identified and confirmed by 16S rRNA sequencing approach. Two strains were obtained from the American Type Culture Collection (ATCC) which were *P. aeruginosa* ATCC BAA-2108 and *P. aeruginosa* ATCC27853. All *P. aeruginosa* were cultured on Mueller Hinton Broth (MHB) or Tryptic Soy Agar (TSA) when recovered from the stock cultures.

### Antibiotic Susceptibility Test (AST)

Antibiotic susceptibility test (AST) was performed using Kirby-Bauer disk diffusion test. Samples were cultured in Muller-Hinton broth and incubated at 37°C for 18 to 24 hours (Suhaili et al., 2018; Ariffin et al., 2020). The optical density (OD) 0.5 were adjusted using sterile distilled water at $600_{nm}$ to form a standardized bacterial suspension. Using sterile cotton swab, the bacterial suspension was inoculated onto Mueller-Hinton Agar (MHA) and an appropriate antimicrobial-impregnated disks were placed on the surface of the agar. The antibiotics used were norfloxacin (10 µg), ciprofloxacin (10 µg), doripenem (10 µg), meropenem (10 µg), oxacillin (1 µg), cefoxitin (30 µg), penicillin (10 µg), kanamycin (30 µg), vancomycin (5 µg), erythromycin (30 µg) and linezolid (30 µg). The plates were inverted and incubated at 37°C for 24 hours before observing the inhibition zone. An interpretive category of Susceptible (S), Intermediate (I) or resistance (R) of the isolates to the classes of antibiotics were determined according to the Clinical & Laboratory Standard Institute: Performance Standard for Antimicrobial Disk Susceptibility Test 2018 (CLSI, 2018). The assay was performed in triplicate with mean±SD calculated.

**Biofilm Formation Assay**

This assay was performed using the crystal violet staining method in 96-well plate. The samples were grown in Mueller-Hinton broth and incubated at 37°C overnight. About 1 mL of bacteria inoculums were aseptically removed to be diluted in sterile Mueller-Hinton broth in order to obtain the optical density (OD) of 0.1 at 600 nm. About 100 µL of the adjusted inoculums were transferred into sterile 96-well plates. Broth without bacteria was prepared as a negative control. The incubation was done at 37°C for 7 days. Then, the media were removed by using micropipette and washed three times with sterile distilled water to remove free-floating planktonic bacteria. The plate was air dried for 15 to 30 minutes. The biofilms were stained with 100 µL of 0.1% crystal violet and incubate for 10 minutes. The crystal violet was removed using micropipette followed by washing step with phosphate-buffered saline (PBS) three times to remove excess crystal violet stain. About 100 µL of 95% ethanol was added into each well to detach the biofilms. The solubilized biofilms were measured by the micro plate reader at 570 nm wavelength (Al-Kafaween et al., 2019). The following formulas were used to classify the biofilm formation. Non-adherent [NA=OD≤ODC)], weak adherent [WA=ODC<OD≤(2×ODC)], moderate adherent [MA=(2×ODC)<OD≤(4×ODC)] and strong adherent [SA=(4×ODC)<OD)] (Nyenje et al. 2013).

**DNA extraction**

Genomic DNA was extracted using Wizard Genomic DNA Purification Kit (Promega), following the methods outlined for Gram-negative bacteria. About 1 ml of an overnight culture was centrifuged at 13,000–16,000 × g for 2 minutes to pellet the cells. About 600 µl of Nuclei Lysis Solution was added to the tube and gently pipette to resuspend the mixtures. The bacterial suspension was incubated at 80°C for 5 minutes to lyse the cells and cool to room temperature. About 3 µl of RNase Solution was added to the cell lysate and mixed by inversion for 2-5 times. The cell lysate was incubated at 37°C for 15–60 minutes and let it cool to room temperature. About 200 µl of Protein Precipitation Solution was added to the RNase-treated cell lysate and mix by vigorous vortex at high speed for 20 seconds. The samples were incubated on ice for 5 minutes and centrifuged at 13,000–16,000 × g for 3 minutes.

The supernatant containing the DNA was transferred to a clean 1.5 ml centrifuge tube containing 600 µl of room temperature isopropanol. The solution was mixed by inversion until the thread-like strands of DNA form a visible mass. The tubes are centrifuged at 13,000–16,000 × g for 2 minutes and the supernatant was discarded. The tubes were drained on clean absorbent paper. To wash the DNA pellet, 600µl of room temperature 70% ethanol was added and gently invert the tube several times. The tubes were centrifuged at 13,000–16,000 × g for 2 minutes. The tubes were drained on a clean absorbent paper and the pellet were air-dried for 10 -15 minutes. Next, about 100 µl of DNA Rehydration Solution was added to the tube to rehydrate the DNA by incubating at 65°C for 1 hour. The DNA is stored at -20°C until further used. The isolated DNA were quantified using spectrophotometer at an absorption of 260 nm. The purity of the DNA is assessed by measuring the ratio of the absorbance at 260 and 280 nm. Ratio of 1.7 to 2.0 is considered as acceptable ratio for all the samples.

**Polymerase Chain Reaction**.

PCR amplification is conducted to identify the presence of biofilm-related genes; *pelA, pslA* and *ndvB* in *P. aeruginosa* isolates. The primers sequence for each of the genes was stated in Table 1. For Polymerase Chain Reaction (PCR), the materials needed were 5X PCR buffer, 25 µM Magnesium Chloride, 10 µM dNTPs mix, 10 µM forward and reverse primers, 1 U Taq polymerase, 1 µg DNA sample and sterile distilled water for a total final reaction of 25 µL. The PCR was carried out using a PCR machine from Applied Biosystems® Veriti® 96-Well Thermal Cycler under the following conditions as stated in Table 2 for each gene. The PCR products were separated on 1 % agarose gel electrophoresis at 80 V, 400 A for 1 hour.

**Table 1: PCR amplification primers for *pslA*, *pelA* and *ndvB* genes.**

| Gene | Primer sequence (5' – 3') | References |
|---|---|---|
| PslA | Forward: TCCCTACCTCAGCAGCAAGC<br>Reverse:<br>TGTTGTAGCCGTAGCGTTTCTG | Pournajaf et al. (2018) |
| PelA | Forward:<br>CATACCTTCAGCCATCCGTTCTTC<br>Reverse: CGCATTCGCCGCACTCAG | |
| ndvB | Forward: GGCCTGAACATCTTCTTCACC<br>Reverse: GATCTTGCCGACCTTGAAGAC | Beaudoin et al., (2012) |

**Table 2: PCR amplification profiles for three genes *pelA*, *pslA* and *ndvB*.**

| Steps | PCR amplification cycle | | |
|---|---|---|---|
| | ***pelA*** | ***pslA*** | ***ndvB*** |
| Initial denaturation | 95°C (5 min) | 94°C (5 min) | 95°C (2 min) |
| Denaturation | 95°C (1 min) | 94°C (30 s) | 95°C (1 min) |
| Annealing | 60°C (1 min) | 52°C (40 s) | 60°C (1 min) |
| Extension | 72°C (1 min) | 72°C (50 s) | 72°C (1 min) |
| Final extension | 72°C (5 min) | 72°C (7 min) | 72°C (10 min) |
| Cycle | 35 | 35 | 30 |
| References | Ghadaksaz et al., 2015 | Nermine et al., 2019 | Saffari et al., 2017 |

**Data analysis**

Experimental data were analyzed using Excel statistical data analysis. Results in this study were presented as mean of triplicates value with standard deviation (Mean ± SD).

**RESULTS AND DISCUSSION**

*P. aeruginosa* has become one of the opportunistic pathogens associated with many healthcare associated infections which involved multi-drug resistance. Now, this strain has evolved to be a challenge and concerns worldwide. According to Table 3, sample 13(1) showed resistance towards 3 groups of antibiotics including β-lactams (penicillin), glycopeptide (vancomycin) and oxazolidinone (linezolid). This isolate also showed intermediate-resistant levels towards β-lactams (oxacillin) and aminoglycosides (kanamycin) at 20 mm and 16 mm of zone inhibition respectively. Other isolates 00(1) and 66(1) mostly showed susceptible level towards most of the antibiotics group tested and were found resistant towards glycopeptide (vancomycin) at 7 mm and 12 mm respectively. All strains were found resistant towards vancomycin which showed strong resistant towards this antibiotic. Previous study by (Ahmadi et al. 2016) reported that prevalence study on vancomycin-resistant *P. aeruginosa* strains isolated from orthopedic infections patients have been recorded reached 74.41%. *P. aeruginosa* ATCC BAA-2108 and *P. aeruginosa* ATCC 27853 exhibited resistant towards all antibiotics tested except for *P. aeruginosa* ATCC 27853 which displayed susceptible for quinolones (norfloxacin and ciprofloxacin). For this study, distilled water ($dH_2O$) was used as negative control during the assay. Esther et al (2017) and Sheikh et al (2014) described that any bacteria strain which is found resistant towards any 3 groups of antibiotics (carbapenems, fluoroquinolones, penicillins/ cephalosporins and aminoglycosides) is considered as multidrug resistant (MDR) strain. Hence, isolate 13(1), *P. aeruginosa* ATCC BAA-2108 and *P. aeruginosa* ATCC 27853 were considered as multidrug resistant (MDR) strains from this study. Although the rate of multi-resistance strains in the present study was still low, this may be an alarming situation that reflects a threat towards new emerging resistant isolates.

Interestingly from the observation, all three *P. aeruginosa* isolates 13(1), 00(1) and 66(1) were also highly sensitive to the carbapenem group of antibiotics which included doripenem and meropenem. Carbapenems resistance among Gram-negative pathogens is now become an ongoing public-health concerns globally. This type of antimicrobial resistant could spread rapidly and cause outbreaks. Prevalence study by Javiya et al. (2008) in the tertiary care hospital in Gujarat, India exhibited about 19.64% of the *P. aeruginosa* isolates were observed resistant against carbapenems. The resistance to carbapenems, especially in *P. aeruginosa* were resulted from reduced levels of drug accumulation, increased expression of efflux pump and due to the production of metallo-β-lactamases (MBL (Kurukawa et al. 1999; Navneeth et al. 2002 and Gupta et al. 2006).

Biofilm-forming ability in *P. aeruginosa* provides the bacteria with resistance towards antimicrobial agents. It causes difficulties in the eradication and control of infection once the pathogen colonizes in its host. Biofilm components in *P. aeruginosa* composed of three exopolysaccharides such as *psl*, *pel* and alginate (Franklin et al. 2011). A previous study carried out on mutant lacking alginate lead to the development of weak biofilms. While deletion of *psl* and alginate also lead to overproduction of pel and loss some important characteristic in biofilm structure. These indicate the importance of these exo-polysaccharides in the early stages of biofilm formation.

**Table 3: Diameter of inhibition zone of antibiotics against *P. aeruginosa* isolates.**

| Classification group | Drugs[a] | Diameter of Inhibition Zone (mm) | | | | | Susceptibility (S/I/R)[b] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 13(1) | 00(1) | 66(1) | ATCC BAA-2108 | ATCC 27853 | 13(1) | 00(1) | 66(1) | ATCC BAA-2108 | ATCC 27853 |
| Carbapenems S (≥19 mm) I (16-18 mm) R (≤15 mm) | DOR (10µg) | 31.0 | 38.0 | 45.0 | 3.0 | 13.7 | S | S | S | R | R |
| | MEM (10µg) | 30.0 | 35.0 | 43.0 | 3.3 | 8.7 | S | S | S | R | R |
| β-lactams S (≥21 mm) I (17-20 mm) R (≤16 mm) | FOX (30µg) | 21.0 | 30.0 | 34.0 | 0.0 | 0.0 | S | S | S | R | R |
| | OX (1µg) | 20.0 | 25.0 | 34.0 | 0.0 | 0.0 | I | S | S | R | R |
| | P (10µg) | 13.0 | 37.0 | 42.0 | 0.0 | 0.0 | R | S | S | R | R |
| Quinolones S (≥17 mm) I (13-16 mm) R (≤12 mm) | NOR (10µg) | 17.0 | 24.0 | 32.0 | 5.0 | 27.0 | S | S | S | R | S |
| | CIP (10µg) | 27.0 | 27.0 | 36.0 | 0.0 | 36.0 | S | S | S | R | S |
| Aminoglycosides S (≥17 mm) I (13-16 mm) R (≤12 mm) | K (30µg) | 16.0 | 17.0 | 27.0 | 0.0 | 0.0 | I | S | S | R | R |
| Glycopeptide S (≥17 mm) R (≤16 mm) | VA (5µg) | 12.0 | 7.0 | 12.0 | 0.0 | 0.0 | R | R | R | R | R |
| Macrolide S (≥23 mm) I (14-22 mm) R (≤13 mm) | E (30µg) | 29.0 | 23.0 | 33.0 | 0.0 | 0.0 | S | S | S | R | R |
| Oxazolidinone S (≥21 mm) R (≤20 mm) | LZD (30µg) | 18.0 | 26.0 | 37.0 | 0.0 | 0.0 | R | S | S | R | R |
| -ve control | dH20 (control) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | N.D. | N.D. | N.D. | N.D. | N.D. |

[a]DOR: doripenem; MEM: meropenem; FOX: cefoxitin; OX: oxacillin; P: penicillin G; NOR: norfloxacin; CIP: ciprofloxacin; K: kanamycin; Va: vancomycin; E: erythromycin; LZD: linezolid
[b]S : susceptible; I: intermediate; R: resistance
ATCC BAA-2108: *P. aeruginosa* (resistance to imipenem**);** ATCC 27853*: P. aeruginosa* (wild-type

Quantitative assessment of biofilm formation was performed by the colorimetric microtiter plate assay as described previously by Al-kafaween et al. (2019) with slight modifications. All strains were considered as biofilm producers as shows in Table 4. For this biofilm assay, two media were used which were Mueller-Hinton broth and tryptic soy broth with 2% sucrose added. This study aims to evaluate carbon source factors in promoting biofilm development. From the assay, isolate 66(1) form the weakest biofilm followed by isolate 00(1) and 13(1). Moreover, *P. aeruginosa* ATCC BAA-2108 and *P. aeruginosa* ATCC 27853 displayed strong adherent for biofilm formation in Mueller-Hinton broth. Among these two strains, *P. aeruginosa* ATCC BAA-2108 possesses strong biofilm formation in both media tested. The strain also has been proved to be resistant towards all antibiotics groups tested in antibiotic susceptibility test (AST). Thus, this finding supports that strong biofilm formation leads to high resistant ability in the bacteria strain.

**Table 4: Optical density (OD) and biofilm development classification of different *P. aeruginosa* isolates.**

| Samples | Mueller-Hinton broth | | Tryptic Soy broth + 2% sucrose | |
|---|---|---|---|---|
| | OD | Biofilm development classification[a] | OD | Biofilm development classification[a] |
| 13(1) | 0.497 | M.A. | 0.732 | M.A. |
| 00(1) | 0.244 | W.A. | 0.593 | M.A. |
| 66(1) | 0.221 | W.A. | 0.460 | M.A. |
| ATCC 27853 | 1.084 | S.A. | 0.535 | M.A. |
| ATCC BAA-2108 | 2.467 | S.A. | 1.403 | S.A. |

[a]N.A.: non-adherent; W.A.: weak adherent; M.A.: moderate adherent; S.A.: strong adherent.

Moreover, for the isolate 00(1) and 66(1), both showed moderate adherent (MA) for biofilm formation when grown on a rich media like tryptic soy broth supplemented with 2% sucrose as compared to weak adherent (WA) growth in the Mueller-Hinton broth. Carbon source is one of the factor influence the ability of biofilm formation in these *P. aeruginosa* isolates (Aboulkacem et al., 2014). Wijesinghe et al. (2019) stated that media with high nutrient composition enhanced the growth of biofilms. The presence of glucose (0.25%) in the Tryptic Soy broth has been reported to enhance biofilm formation (Nyenje et al., 2013 and Ciofu et al. 2017). This was in agreement with the increased biofilm formation in a few *P. aeruginosa* isolates 13(1), 00(1) and 66(1) in tryptic Soy broth (Figure 1).

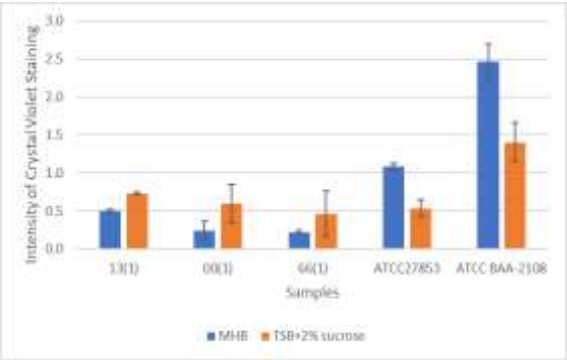


**Figure 1: Comparison of biofilm formation in different media used among the *P. aeruginosa* isolates.**

In some cases, nutrient deprivation drives bacteria to form biofilm as a defence and stress response towards the environment factors (Jefferson, 2004). When carbon source is depleted, it causes carbon limitation and bacteria usually forms and produces biofilm in order to reserve nutrients in the biofilm matrix (Aboulkacem et al. 2014).

Furthermore, studies on genes involved in the regulation of biofilm formation are very important to carried out. All three genes *pelA*, *pslA* and *ndvB* were presented in the isolate 13(1), 00(1), 66(1) and *P. aeruginosa* ATCC BAA-2108 which were previously identified as biofilm-forming strains (Figure 2 and Figure 3). This result revealed significant correlation between the biofilm forming ability and the presence of relevant genes as supported by biofilm formation assay. Thus, this suggest that the three genes are involved in the biofilm development. Moreover, two bands showed on the amplification of *pslA* gene might be due to primers were non-specific for *P. aeruginosa* ATCC BAA-2108 strain, thus, leads to non-specific PCR amplification products as showed in Figure 3.

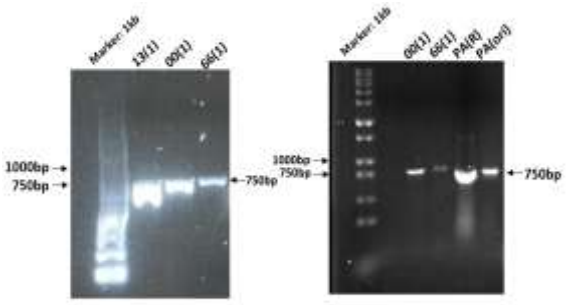


**Figure 2: PCR amplification of *pelA* gene on all *P. aeruginosa* isolates. PA(R): *P. aeruginosa* ATCC BAA-2108; PA(ori): *P. aeruginosa* ATCC27853.**

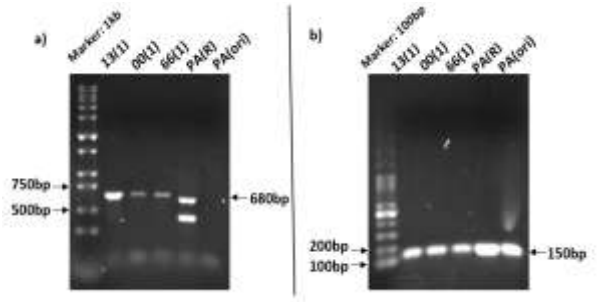


**Figure 3: PCR amplification of biofilm-encoding genes, (a) *pslA* gene; (b) *ndvB* gene. PA(R): *P. aeruginosa* ATCC BAA-2108; PA(ori): *P. aeruginosa* ATCC27853**

Marmont et. Al. (2017) mentioned that *pelA* interacts with *pelB* in *Pseudomonas aeruginosa* allows the localization of *pelA* to the outer membrane which is essential for the assembly of polysaccharide. Colvin et al. (2012) demonstrated that *pelA* deletion mutant formed significantly reduced biofilm as compared to the wild type. Another gene which is also involved in the resistance mechanism in biofilm strain is *ndvB* gene. This gene encodes for a glucosyltransferase enzyme which is important for the synthesis of cyclic-β 1,3 glucans which contribute to antibiotic resistance in biofilms specifically aminoglycoside by sequestering them away from their cellular targets (Zhang et al. 2013).

Interestingly, for *P. aeruginosa* ATCC 27853, two genes (*pelA* and *ndvB*) were presence in this strain except for *pslA* gene. *P. aeruginosa* ATCC 27853 was able to develop robust biofilm despite lacking the *pslA* gene. Colvin et al. (2012) stated that *P. aeruginosa* can use either *psl* or *pel* as the primary biofilm matrix polysaccharide. *Pel* and *psl* both serve redundant functions as structural scaffolds in mature biofilms depending on the bacteria strain. Certain strain relies primarily on either *pel* or *psl* for its biofilm development (Colvin et al. 2012). As reported by Ghafoor et al. (2011), *P. aeruginosa* UCBPP-PA14 strain has been identified use *pel* polysaccharide as a dominant polysaccharide in its biofilm matrix (Ghafoor et al., 2011). Besides that, *pslA* also involved in the biosynthesis of *psl* polysaccharide which plays an important role in adhesion and surface attachment as it provides cell–cell and cell–surface interactions (Colvin et al. 2013). Study by Jackson et al. (2004) also proved that disruption of *pslA* and *pslB* genes led to a profound biofilm initiation deficiency.

The capacity of biofilm formation despite of the absence of biofilm genes also indicates other factors might play the important role in biofilm development especially in *P. aeruginosa*. As stated by Moradali et al. (2017), one of the factor is genetic determinants which participate in biofilm matrix formation in *P. aeruginosa* (Friedman et al. 2004; Müsken et al. 2010 and Moradali et al. 2017). In contrast, the presence of biofilm related genes without biofilm formation may be due to chromosomal mutations in different regulatory systems which affect the production of functional biofilm-associated proteins.

## CONCLUSION

As conclusion, *P. aeruginosa* ATCC BAA-2108 showed strong resistant against the antibiotics group tested as compared to the isolate 13(1), 00(1), 66(1) and *P. aeruginosa* ATCC 27853 based on antibacterial disc diffusion assay. Besides that, from this study, all *P. aeruginosa* isolates 13(1), 00(1) and 66(1) and both *P. aeruginosa* ATCC strains (BAA-2108 and ATCC 27853) were classified as biofilm-forming strains as supported by the biofilm formation assay. Moreover, the presence of biofilm encoding genes (*pelA*, *pslA* and *ndvB*) on these strains further proved that these genes are involved in the development of biofilm.

## CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

## ACKNOWLEGEMENT

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## AUTHOR CONTRIBUTIONS

NAMAL performed the experiments, participated in data analysis and contributed to manuscript writing. WA performed the sampling and participated in data analysis. NA participated in design of the study, contribute in the manuscript writing and critically reviewed the manuscript. AK contributed to the study design and manuscript review. RMI and AMA participated in manuscript review. All authors read and approved the final version.

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