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ORIGINAL ARTICLE

Biofilm production by *Pseudomonas aeruginosa* Clinical Isolates and its Relationship with Pseudomonas Quinolone Signal *(pqsA*) Gene and Antibiotic Resistance

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P. aeruginosa, biofilm, antibiotic resistance, MBEC, MIC, pqsA

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ABSTRACT

Background*: Biofilms are complex microbial communities anchored to biotic or abiotic surfaces. They contribute to more than 80% of hospital-acquired infections. Pseudomonas aeurginosa (P. aeurginosa) is an important pathogen able to form biofilm which is regulated by quorum sensing molecules including pseudomonas quinolone signal (pqs).* ***Objectives:*** *The present work aimed to study the ability of different P. aeurginosa clinical isolates to produce biofilm and their association with Pqs A gene and antibiotic resistance.* ***Methodology:*** *This study was conducted on 30 isolates of P. aeruginosa obtained from different clinical samples. Detection of antibiotic susceptibility was done by disc diffusion method. Detection of biofilm formation was done using microtitre plate assay. Minimal inhibitory concentration (MIC) and Minimal biofilm eradication concentration (MBEC) were done only for biofilm forming isolates. Detection of Pqs A gene was done using conventional PCR.* ***Results:*** *17 out of 30 (57%) isolates were biofilm producers. Antibiotic resistance was higher among biofilm producing than non biofilm producing isolates. There was statistically significant difference between MIC and MBEC of meropenem and amikacin. There was statistically significant association between biofilm production and Pqs A gene.* ***Conclusion:*** *Biofilm producing strains have high resistance to antibiotics and Pqs A gene has a significant role in biofilm production. Thus, it is recommended to detect MBEC rather than MIC to antimicrobials for treatment of biofilm associated infections and to study the effect of pqs inhibition on biofilm control*

INTRODUCTION

Bacteria exist, in most environments, as complex organized communities of sessile cells embedded within a self-produced matrix. These communities are known as biofilms [1,2]. The microorganism efficiently attaches itself to a substratum for growth and homogenous biofilm development [3,4]. Organisms in biofilms, exhibit different physiology and phenotype in comparison to their planktonic forms[3,4]. Biofilms provide a physical barrier against antimicrobial agents and host immune responses [6,7]. Thus, biofilms render pathogenic microorganisms difficult to eradicate and contribute to localized or systemic chronic infections[8].

They contribute to more than 80% of hospital-acquired and community acquired infections 5.Biofilm associated microbial infections include urinary tract infections, catheter related infections, formation of dental plaque, gingivitis and cystic fibrosis[2].

Pseudomonas aeruginosa (*P.aeruginosa*) is considered to be one of the most important pathogen causing biofilm on human host and is the leading cause of diverse infections including pneumonia, wound and urinary tract infections 9. *P.aeruginosa* communicate with each other by quorum-sensing system mediated by the two chemically distinct classes of signal molecules, the **N**-acylhomoserine lactones and the 4-alkyl-quinolones (AQs) 10,11.Pseudomonas quinolone signal (PQS) is the most active signal molecule in this group. PQS signaling is pleiotropic, regulating production of pyocyanin, elastases, rhamnolipids and lectin , as well as biofilm formation and motility 12. Synthesis of AQs depends on the pqsABCDE locus, which is responsible for generating multiple 4-quinolones 13. The first step of the 4-quinolones synthesis pathway is the generation of the pqsAgene product [14].

The aim of this study was to determine the ability of different *P. aeruginosa* clinical isolates to produce biofilm and the association of biofilm production with the presence of the pqsA gene and antibiotic resistance.

METHODOLOGY

This study was conducted at Ain Shams University Hospitals during the period from July 2015till May 2016. Thirty isolates of *P. aeruginosa* were collected from different clinical samples: 16 were isolated from pus, 8 were isolated from sputum and 6 were isolated

from urine. Identification of the isolated strains was done according to Collee et al. 15 based on colonial morphology, microscopic examination of Gram stained films and biochemical reactions.

Antimicrobial susceptibility testing by Disc diffusion method:

Antibiotic susceptibility of all isolated organisms was done by disc diffusion method, using Muller-Hinton agar plates (Oxoid, U.K). The bacterial suspension wasused as the inoculum at a McFarland no. 0.5(BioMérieux, France). Antibiotic discs used in this study included piperacillin, ceftazidime ,ciprofloxacin, levofloxacin ,amikacin, gentamicin and meropenem (Bioanalyse, Turkey). After overnight incubation results were reported and interpretation was done according to clinical and laboratory standards institute CLSI guidelines [16]. Multidrug resistance (MDR) was defined as resistance to ≥3 classes of antipseudomonal agents (penicillins/cephalosporins, carbapenems, quinolones, and aminoglycosides) [17].

Biofilm formation test:

The isolated organisms were tested for their ability to form biofilm, according to Stepanovic et al. [18]. Flat-bottomed 96-well clear polystyrene tissue culture treated microtitrplate (MP) with a lid (TPP-Switzerland) were inoculated with 200 µl of a bacterial suspension in corresponding to 0.5 McFarland (with further 1:100 dilution). After 24 h incubation at 37°C, the contents of each well were removed by decantation and each well was washed three times with 300 µl of sterile saline. The remaining attached bacteria were heat-fixed by exposing them to hot air at 60°C for 60 minutes in Fisher isotemp incubator, then150 µl crystal violet (2%) stain was added to each well. After 15 min, the excess stain was rinsed off by decantation, and the plate was washed, 150 µl 95% ethanol was added to each well, and after 30 min, the optical densities (OD) of stained adherent bacterial films were read using a microtiter-plate reader (Tecan Sunrise remote Austria) at 620 nm. The average OD values were calculated for all tested strains and negative controls, the cut-off value (ODc) was established. It is defined as a three standard deviations (SD) above the mean OD of the negative control: ODc=average OD of negative control + (3×SD of negative control). Final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value (OD= average OD of a strain - ODc); ODc value was calculated for each microtiter plate separately. When a negative value was obtained, it was presented as zero, while any positive value indicated biofilm production. For easier interpretation of the results, strains were divided into the following categories: 1. Non biofilm producer (0) OD ≤ODc 2. Weak biofilm producer (+ or 1) = ODc

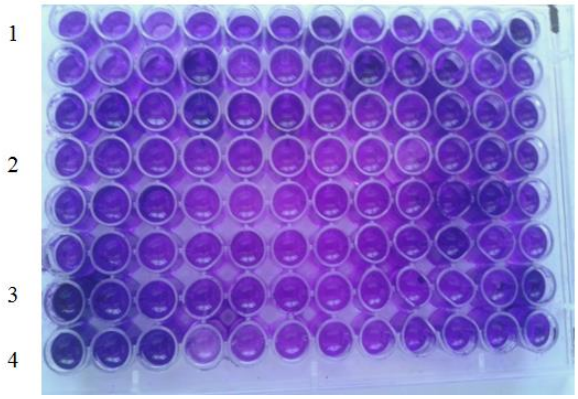

Fig. 1: MTP inoculated with thirty isolates each occupying three wells and six wells for negative control.

1. Non biofilm producer
2. Weak biofilm producer
3. Moderatebiofilm producer
4. Strong biofilm producer
5. Negative control.

Antibiotic susceptibility of biofilm forming isolates:

Amikacin and meropenem were selected to measure minimal inhibitory concentration (MIC) and minimal biofilm eradication concentration (MBEC) as the biofilm forming strains were commonly sensitive to them.

Antibiotic susceptibility of planktonic cells on microtiter plate:

MIC was determined by broth microdilution using 96 wells MTP and results were interpreted according to CLSI guidelines [16]. antibiotic solution was prepared following the manufacturer's guidelines stock solutions of antimicrobial agents.. MIC was measured as the lowest concentration of the antimicrobial agent that inhibited the growth ofthe microorganism being tested as detected by lack of visual turbidity, matching with a negative control included with the test. (figure1)

Table 1: Antibiotics used for MIC, interpretive standards and concentrations used.

Antibiotics	*MIC Interpretive Criteria (µg/ml)*			*Concentrations used(µg/ml)*
	Sensitive	**Intermediate**	**Resistant**	
Amikacin	≤ 16	32	≥ 64	128- 0,25
Meropenem	≤ 2	4	≥ 8	16-0,125

Antibiotic susceptibility of sessile cells on microtiter plate:

MBEC of sessile cells is the minimum antibiotic concentration at which bacteria failed to re-grow. It is measured according to Passerini de Rossi et al., 19 and compared to the MIC of their planktonic counterpart as follows: One hundred µl of the standardized inoculum, as described in biofilm formation, were added to each wellof a 96-well MTP and incubated at 37˚C for 24 h. The medium was then discarded; the wells were washed with saline. One hundred µl of the antibiotics at two fold dilutions was added to the established biofilms. Following overnight incubation, wells were then washed with saline and filled with 100µl of broth. The viability of the biofilm was determined after 24 h of incubation at 37˚C visually through turbidity of broth. MBEC was read as the minimum antibiotic concentration at which bacteria failed to re-grow.
Sterility controls and antibiotic-free controls were included in all experiments (figure 2).

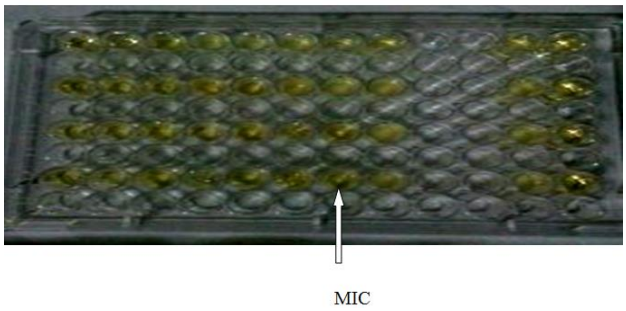

Fig. 2: Meropenem susceptibility of planktonic cells on MTP.

Detection of pqsA gene:

The isolated strains were tested for presence of pqsA gene using conventional Polymerase chain reaction (PCR) technique according to Maita and Boonbumrung [9].

- DNA extraction was done using QiagenDNeasy (Qiagen, USA), for DNA extraction from bacterial cultures according to manufacture instructions [20].
- Amplification of pqsA gene using polymerase chain reaction: we used thermal cycler (Biosystems, USA), Taq PCR Master Mix (Qiagen, USA) and a pair of primers for Pqs A gene (Qiagen, USA) (Table 2).

Table 2: Primer sequence used in the study.

Gene	*Primer direction*	*Primer sequence*	*Length of Primer (bp)*	*Size of Amplified Product (bp)*
Pqs A	Forward	5'- CCCGATACCGCCGTTTATCA -3'	20	448
	Reverse	5'AACCCGAGGTGTATTGCAGG -3'	20	

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- Detection of the amplified product using agarose gel electrophoresis (Figure 3).

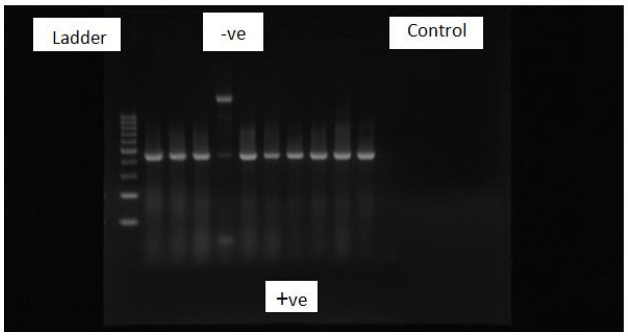

Fig. 3: A garose gel showing the results of some isolates.

Statistical Analysis:

Statistical analysis was done using the Statistical Package for Social Sciences (SPSS) version 17 as follow: Frequency number and percentage for qualitative data, Chi-square test ($\chi 2$) used to compare qualitative variables. Results were considered significant when p value was ≤ 0.05.

RESULTS

In this study 30 *P. aeruginosa* isolates were obtained from urine, pus and sputum. Regarding antibiotic susceptibility,the isolates were most sensitive to meropenem (77%) followed by amikacin (67%), while they were least sensitive to piperacillin (27%) and ceftazidime (7%) (figure 4).

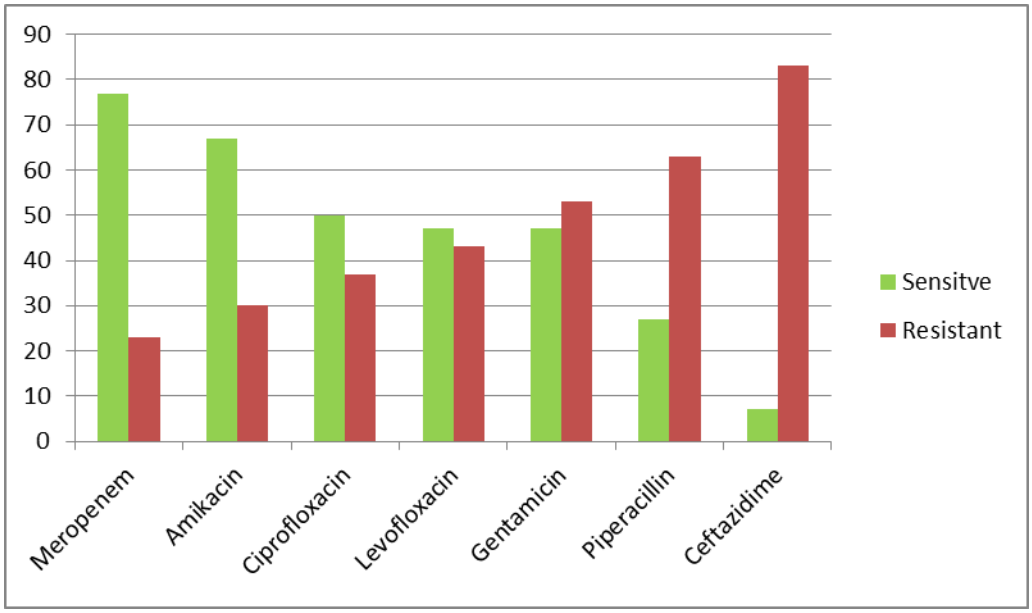

Fig. 4: Antibiotic susceptibility results of *P. aeruginosa* isolates.

As regard biofilm production ; 17 isolates (57%) were positive biofilm producers: 2 isolates (7%) were strong biofilm producers, 8 isolates (27%) were moderate biofilm producers and 7isolates (23%) were weak biofilm producers (figure 5).

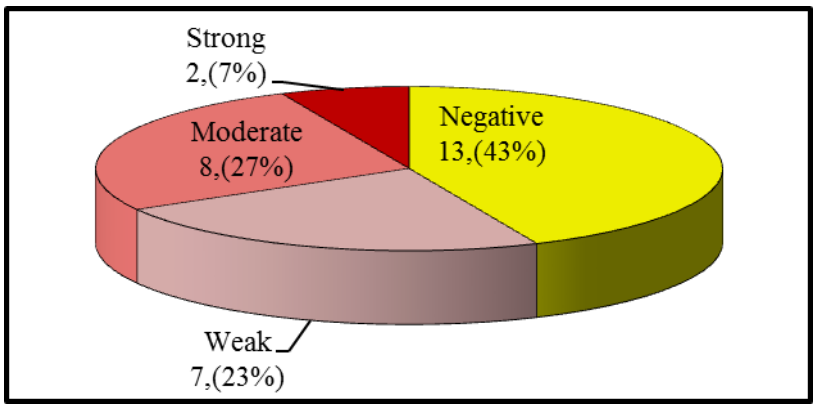

Fig. 5: Biofilm production among different isolates.

The Antibiotic resistance to all used antibiotics were higher among biofilm producing than non biofilm producing strains (table 3 and figure 6.)

Table 3: Difference between antibiotics resistance among biofilm producing and non biofilm producing strains.

Antibiotics resistance	*Biofilm production*		*Chi-square*	
	Positive(17) N (%)	**Negative(13) N (%)**	**X2**	**P-value**
Meropenem	6(35%)	1(8%)	3.471	0.062
Gentamycin	10(59%)	6(46%)	0.476	0.490
Amikacin	6(35%)	3(23%)	1.871	0.392
Ceftazidime	15(88%)	10(77%)	3.584	0.167
Levofloxacin	8(47%)	5(38%)	0.503	0.777
Pipracillin	11(65%)	8(61%)	0.281	0.869
Ciprofloxacin	7(41%)	5(38%)	5.168	0.075

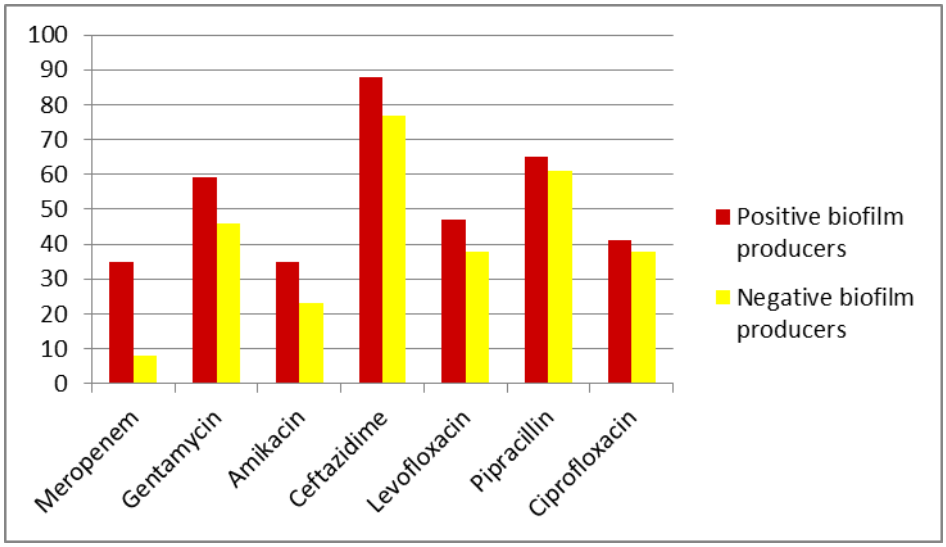

Fig. 6: Difference between antibiotics resistance (disc diffusion methods) among biofilm producing and non biofilm producing strains.

Meanwhile, MDR was higher among biofilm producing than non biofilm producing strains (table 4).

Table 4: Difference between MDR among biofilm producing and non biofilm producing strains.

Antibiotics resistance	*Biofilm production*		*Chi-square*	
	Positive(17) N (%)	**Negative(13) N (%)**	**X2**	**P-value**
MDR	11(65%)	6 (46%)	1.035	0.309

Moreover, there was a highly significant differences between MIC and MBEC of meropenem and amikacin ($p<0.01$) (table 5 and 6, figure 7 and 8)

Table 5: Comparison between MIC and MBEC in meropenem sensitive biofilm producing isolates.

Test result / *Antibiotic*	*MIC(0.125-4ug/ml)*			*MBEC(8-16ug/ml)*			*X2*	*P value*
	S No(%)	**In No(%)**	**R No(%)**	**S No(%)**	**In No(%)**	**R No(%)**		
Meropenem	11 (100%)	0	0	0	0	11 (100%)	22	<0.001

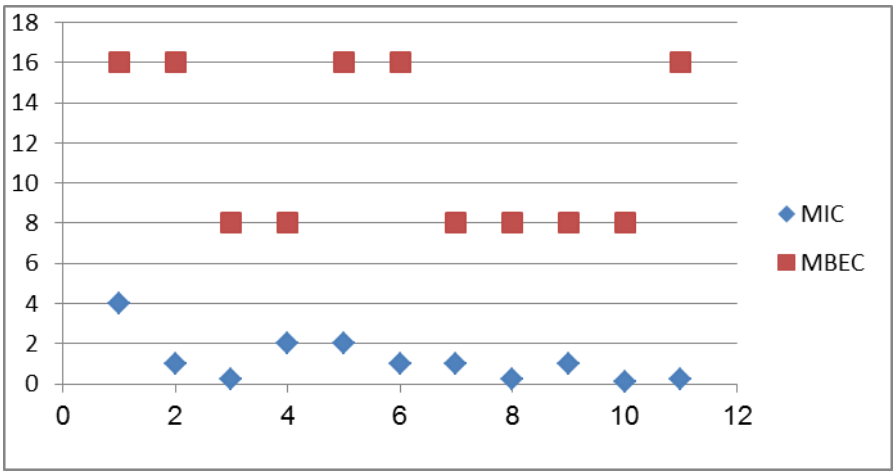

Fig. 7: Comparison between MIC and MBEC in meropenem sensitive biofilm producing isolates.

Table 6: Comparison between MIC and MBEC in amikacin sensitive biofilm producing isolates.

Test result / *Antibiotic*	*MIC(0.25-32ug/ml)*			*MBEC(≥128ug/ml)*			*X2*	*P value*
	S No(%)	**In No(%)**	**R No(%)**	**S No(%)**	**In No(%)**	**R No(%)**		
Amikacin	9 (90%)	1 (10%)	0	0	0	10 (100%)	20	<0.001

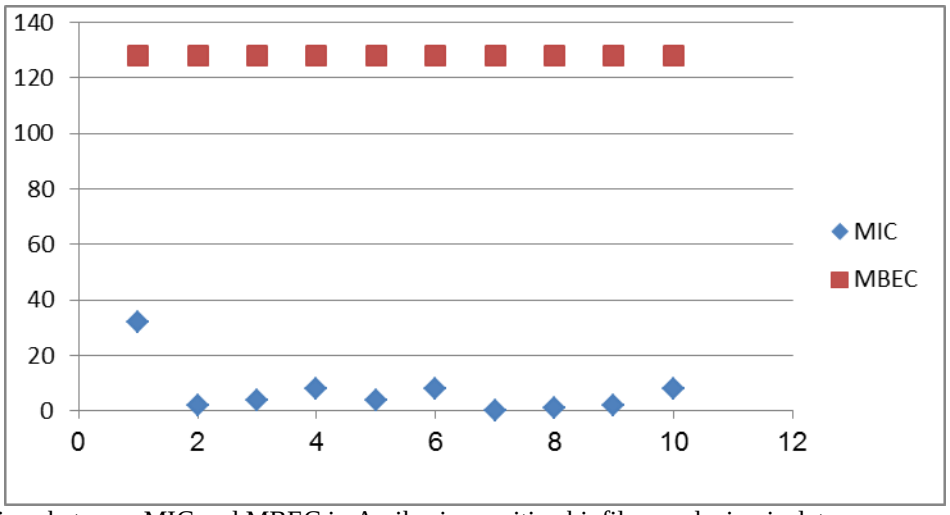

Fig. 8: Comparison between MIC and MBEC in Amikacin sensitive biofilm producing isolates.

Pqs A gene was found in 90% (27/30) of isolates. All the 17 positive biofilm producers had the Pqs A gene compared to 77% of negative producers and this difference was statistically significant (table 7).

Table 7: Association between biofilm production and PqsA gene.

Biofilm production	*Pqs A gene*					*Chi-square*	
	Negative		**Positive**		**Total**		
	N	**%**	**N**	**%**	**N**	**X2**	**P-value**
Negative	3	23%	10	77%	13(100%)	5.46	0.019
Positive	0	00%	17	100%	17(100%)		

DISCUSSION

P.aeruginosa has become an important cause of gram-negative infections, especially in immunocompromized patients. It is the most common pathogen isolated from patients who have been hospitalized longer than 1 week [21]. The majority of *P.aeruginosa* strains are able to produce biofilms that are responsible for chronic infections and antibiotic resistance [22]. In this study, we attempted to determine the ability of different *P. aeruginosa* clinical isolates to produce biofilm and the association of biofilm production with the presence of the pqsA gene and antibiotic resistance.

In the current study, the lowest antibiotic resistance of *P. aeruginosa* isolates was to meropenem 23% (7/30) followed by amikacin 30% (9/30). Different results were detected by Maita and Boonbumrung 9 who reported that 36% (49/136) of *P.aeruginosa* isolates were resistant to meropenem and13.2% (18/136) were resistant to amikacin. The highest resistance in the current study was to ceftazidime 83% (25/30) followed by piperacillin 63% (19/30). Different results were detected by Ghanbarzadeh et al.23 who reported that 85.4% (12s) of *P.aeruginosa* isolates were resistant to pipracillin and 82.6% (119/144) were resistant to ceftazidime. High level of resistance to ceftazidime and piperacillin may be due to their wide use in our hospitals. The difference in results between studies may be attributed to the different antibiotic policies implemented in each country.

Regarding biofilm production, 57% (17/30) of clinical isolates were positive biofilm producers; 7% (2/30) produced strong biofilm, 27% (8/30) produced moderate biofilm and 23% (7/30) produced weak biofilm.

Several studies reported different rates of biofilm production by *P.aeruginosa* isolate. Maita and Boonbumrung [9] reported that 60% (82/136) of *P. aeruginosa* isolates obtained from different clinical samples were strong biofilm producers, 11% (14/136) were moderate biofilm producers and 7% (9/136) were weak biofilm producers. Kaur and Wankhede 26 reported that 45% (27/60) of *P. aeruginosa* isolated from different clinical samples were good biofilm producers and 20% (12/60) were weak biofilm producers .The difference in rates of biofilm production may be attributed to difference in isolates capacity to form biofilm, the primary number of cells that succeeded in adherence, the differences of quality and quantity of autoinducers that produced from each isolate [24] and the pattern of motility determined by type IV pili and flagella affect degree of *P. aeruginosa* biofilms [25].

The difference in results between different studies may be attributed to type and also the number of samples collected in each study may play a role. Difference in isolates capacity to form biofilm [24].

Meanwhile, we found that antibiotic resistance to all used antibiotics were higher among biofilm-producing than non-biofilm producing isolates but the difference was statistically non significant. The highest difference was in meropenem (35% versus 8%), gentamycin(59% versus 46%) and amikacin. (35 versus 23%) The lowest difference was in pipracillin(65% versus 61%) and ciprofloxacin (41% versus 38%). A similar result was obtained by Emami et al. 27 who reported that resistance to ceftazidime, gentamicin, tobramycin, piperacillin, imipenem, ciprofloxacin and amikacin were higher among biofilm producing pseudomonas isolates than non-producing isolates . Moreover, Nithyalakshmi et al. [28] found a significant difference in resistant rates between biofilm producer and non-producer *P. aeruginosa* isolates; 68.75% versus 37.5% in ciprofloxacin, 56.25% versus 18.75% in ceftriaxone, 75% versus 10.94% in ceftazidime and 29.1% versus 14.5% in levofloxacin ,while no difference was found in piperacillin and amikacin

Although we found that multidrug resistance (MDR) was higher among biofilm-producing 65% (11/17) than non-biofilm producing isolates 46% (6/13), yet the difference was statistically non significant. Ghanbarzadeh et al.[23] Udokwu [29] and Gurung et al.[30] similarly reported that MDR isolates existed in both biofilm-positive and negative groups, but most of them were significantly associated with the biofilm group. These results showed that biofilm producing isolates have high antibiotic resistance tendency even in their planktonic form. Antibiotic resistance within a biofilm arises from multiple factors, including exopolysaccharide matrix acting as a physical barrier to antibiotic penetration and the creation of an antibiotic gradient throughout the biofilm. In addition, oxygen and nutrient depletion may cause the bacteria to enter a non-growing or stationary phase, which increases resistance to antibiotics such as β-lactams. Exposure of cells within the biofilm to sublethal concentrations of antibiotics further promotes antimicrobial resistance in cells that may then detach from the biofilm and disseminate infections elsewhere [31].

Moreover, biofilm-specific antimicrobial resistance genes not expressed during the planktonic phase have been shown to increase resistance of cells in these sessile communities and the proximity of cells within a biofilm can facilitate plasmid exchange and hence enhance the spread of antimicrobial resistance [6].

However, the non significant association between biofilm production and antibiotic resistance we found in this study may be due to low number of isolates, the method performed for detection of antibiotic resistance (disc diffusion method) that used planknotic not sessile cells or the presence of other mechanisms of resistance other than biofilm formation.

The present study showed a high statistical significant difference between amikacin and meropenem MIC and MBEC (MBEC was 2 to more than 10 fold higher than the MIC). Similar results were obtained by Cernohorska and Votava [32] who found that MBEC was 4 to 533 fold higher than the MIC for all tested antibiotic (amikacin, cefepim, cefoperazon, ceftazidim, ciprofloxacin, meronem, netilmicin, piperacillin-tazobactam and piperacillin). These results confirm that the concentration required to eradicate biofilms is higher than that required to inhibit planktonic cells. The MIC assay is a commonly used method to test antibiotic efficacy because it is quick and reproducible. However, it is not an effective assay for testing antibiotics against adherent biofilm [33].

In the current study,we detected the presence of Pqs A gene in 90% (27/30) of *P.aeruginosa* isolates. All biofilm producing isolates (17/17) had gene compared to 77% (10/13) of non-biofilm producing isolates indicating a significant association with biofilm production.Several previous studies found the same significant association. Maita and Boonbumrung [9] found that 49.1% of biofilm producing isolates had Pqs A gene compared to 21.4% of non-biofilm producing isolates. Meanwhile, Allensen-Holm et al.[34] found that isolates deficient in the pqsA gene generated low amounts of extracellular DNA (e DNA) and produced thin, flat biofilms while mutants over expressed PqsA generated larger amounts of e DNA. Moreover, Müsken et al. [35] reported that pqsA mutants formed flat biofilms without mushroom-shaped structures and lacking the heterogeneity of the wild-type biofilm .

These results confirm that Pqs is necessary for biofilm formation especially the cap portion of the mushroom-shaped structures in *P. aeruginosa* biofilm rather than in initial step, bacterial attachment. This is mediated by regulation of numerous factors including the production of e DNA and rhamnolipid. The e DNA is one of the major matrix components and rhamnolipid

promot motility occurring in the later phase biofilm formation [36].

CONCLUSION

Biofilm producing strains have high resistance to antibiotics even in their planktonic form, Pqs A gene has a significant role in biofilm formation. Measurement of MBEC rather than MIC is recommended to be done for biofilm incriminated infections.

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