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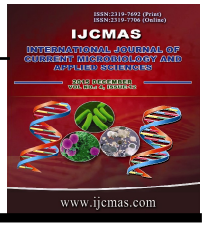

Original Research Article

Efflux Pump PA1874 Gene Expression among *Pseudomonas aeruginosa* Isolates in Zagazig university Hospitals, Egypt

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Keywords

P. aeruginosa, Biofilm, Real time PCR and *PA1874 gene*

A B S T R A C T

PA 1874 is an efflux pump with a role in the antibiotic resistant mechanism among *Pseudomonas aeruginosa* especially biofilms. This work aimed to detect the biofilm formation and its effect on antibiotic susceptibility pattern of *P. aeruginosa* isolated from clinical samples, and to measure the expression of *PA1874* gene among *P. aeruginosa* isolates. This study was conducted on 64 *P. aeruginosa* isolates. Biofilm was measured using tube method. Antibiotic susceptibility of non-biofilms and biofilm +ve isolates and planktonic counterparts for ciprofloxacin, tobramycin and gentamycin was tested using tube microdilution method. Expression of *PA1874* gene was measured using Syper green real time PCR. We found that 26 isolates (40.6%) were biofilm +ve.The biofilm formation was statistically significant high among endotracheal tube aspirate and urine isolates. None of biofilm forming isolates were susceptible to the 3 antibiotics compared to the presence of some susceptible isolates among the planktonic counterpart and non-biofilms. In biofilm isolates, expression of *PA1874* gene was significantly high in biofilm cells more than planktonic counterpart and non biofilms. We concluded that *P. aeruginosa* has high ability of biofilm formation that may be the main cause of antibiotic resistance especially in isolates from artificial devices as urinary catheters and endotracheal tubes and *PA1874 gene* expression is an important mechanism of resistance in *P. aeruginosa* biofilm.

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen and has an extremely versatile lifestyle, its infection is life-threatening for immuno-compromised patients and it is a feared pathogen for burn centers as well as a leading cause of death in cystic fibrosis patients. Furthermore, *P. aeruginosa* is often resistant to treatment and develops antibiotic resistance over time (Sigurdsson *et al*., 2012).

Biofilms are surface-attached microbial communities with characteristic architecture, phenotypic and biochemical properties distinct from their free-swimming, planktonic counterparts. One of the best-known of these biofilm-specific properties is the development of antibiotic resistance that can be up to 1,000-fold greater than planktonic cells. A genetic determinant of this high-level resistance in the Gram-

negative opportunistic pathogen, *P. aeruginosa* was reported. Results indicate that biofilms themselves are not simply a diffusion barrier to these antibiotics, but rather that bacteria within these microbial communities employ distinct mechanisms to resist the action of antimicrobial agents (Mah *et al.*, 2003).

An antibiotic resistant mechanism that has undergone a great deal of study in *P. aeruginosa* is the transport of antimicrobials via efflux pump systems, especially those of the resistance-nodulation-cell division (RND) family. RND efflux pumps have been shown to cause the efflux of a multitude of substrates, including most classes of antibiotics (Gillis. *et al*., 2005).

Resistance-nodulation-division (RND) transporters in *P. aeruginosa* have a wide range of substrates. For instance, MexAB-OprM, MexCD-OprJ, and MexXY-OprM extrude quinolones, macrolides, tetracyclines, β-lactams, and more, while aminoglycosides are more specific to MexXY-OprM (Masuda. *et al.*, 2000).

A novel efflux pump in *P. aeruginosa* was identified to be important for biofilm-specific resistance to a subset of antibiotics. Complete deletion of the genes encoding this pump, *PA1874 to PA1877* (*PA1874-1877*) genes, in an *P. aeruginosa* PA14 background results in an increase in sensitivity to tobramycin, gentamicin, and ciprofloxacin, specifically when this mutant strain was growing in a biofilm (Zhang and Mah, 2008).

This work was conducted to detect the biofilm formation among *P. aeruogionsa* then, study the effect of biofilm on antibiotic susceptibility pattern of *P. aeruginosa* isolated from clinical samples, and to measure the expression of *PA1874 gene* among them.

Materials and Methods

Bacterial Strains

A total number of 64 *P. aeruginosa* isolates was originated from patients hospitalized in intensive care units, and surgery and burn units in Zagazig University Hospitals during the period from August 2013 –March 2014. Isolates were obtained from 186 specimens including catheterized urine, endotracheal aspirates and exudates of burn and septic wound. The specimens were sent to the Lab. of Microbiology & Immunology, Zagazig University Hospitals. The isolates were identified to species level by API 20NE and stored at -70 ° C.

Detection of Biofilm Formation

It was done by tube method according to (Christensen *et al.,* 1982).Briefly, a loopful of test organism was inoculated in 10 ml of TSB with 1% glucose in test tubes. The tubes were incubated at 37°C for 24 h. After incubation, tubes were decanted, washed with PBS then stained with crystal violet (0.1%).The experiment was performed in triplicate. The strain was considered as biofilm positive when there was a visible film lined the wall and the bottom of the tube and considered as a non-biofilm producer if the wall of the glass tube remained unstained (Prasad *et al.,* 2009).

Antibiotic Susceptibility Testing by Broth Microdilution Method

Antimicrobial Agents

Test drugs included gentamycin, tobramycin and ciprofloxacin, obtained from Sigma Company, USA as standard powder. Antibiotics were prepared as stock solutions (5120ug/ml for tobramycin and gentamycin, and 1280ug/ml for ciprofloxacin) in cation-

adjusted Muller Hinton broth (CAMHB) and stored at -70°C (NCCLS, 2007).The antimicrobial agents were serially diluted in CAMHB ranged in concentrations; from 1024 -1 ug for gentamycin and tobramycin and 256- 0.25ug for ciprofloxacin**.**

Biofilm Susceptibility Assay-

The minimal biofilm inhibitory concentrations (MBICs) of the antibiotics were determined according to Černohorská and Votava (2004). The experiments were done in 96-wells polystyrene microtiter plates with round bottoms. An overnight culture was adjusted with TSB to achieve a turbidity equivalent to that of a 0.5 McFarland standard, and then 75µL aliquots of the inoculated media were added to the wells of microtiter plates. The plates were incubated for 24 h at 37°C. The wells were washed three times with phosphate-buffered saline (PBS) under aseptic conditions to remove unattached bacteria and dried in an inverted position. Volumes of 100µL of appropriate two-fold dilutions of the respective antimicrobial agents in CAMHB (512- 1 ug/ml for gentamycin and tobramycin, and 128- 0.25 ug/ml for ciprofloxacin) were transferred into the dried wells with established biofilms. The microtiter plates were incubated for 18–20 hours at 37 ºC, and MBIC was determined, which corresponds to the lowest concentration of antibiotic that inhibits growth of biofilm cells as indicated by absence of visible growth in the wells. A positive control and a negative control were included in all experiments. The experiment was repeated three times.

Non-Biofilm and Planktonic Susceptibility Assay

Minimum inhibitory concentration (MIC) of the planktonic and non biofilm bacterial population was determined by standard broth microdilution method according to NCCLS guidelines (2006). MIC was determined as the lowest concentration of antibiotic at which there is no visible growth**.**

Interpretation

The strains which have tobramycin, gentamicin & ciprofloxacin MIC (or MBIC) values at $\leq$ 4 ug/ml, $\leq$ 4 ug/ml, and $\leq$ 1 ug/ml respectively, were accepted as susceptible and which have MIC (or MBIC) values at $\geq$ 8 ug/ml, $\geq$ 8 ug/ml, and $\geq$ 4ug/ml respectively, were accepted as resistant (NCCLS, 2007).

Real- time Polymerase Reaction

It was done to measure the expression of efflux pump gene *PA1874* in biofilm forming *P. aeruginosa* strains and its planktonic counterparts, and non biofilm forming strains

RNA Extraction and cDNASynthesis-

Total bacterial RNA was isolated from non-biofilm, the planktonic culture and colony biofilms. Briefly, the planktonic and non-biofilm cells were grown in LB broth at 37°Cto early stationary phase (optical density at 600 nm of 0.7), while the biofilm cells were prepared from colony biofilms grown on nutrient agar plates overnight at 37°C followed by another night at room temperature as described previously (Zhang and Mah, 2008). RNA was extracted using IQeasy TM plus CTB RNA Extraction Mini Kit (iNtRON Biotechnology, Inc, Korea) according to manufacturer instructions. Reverse Transcription was done using Maxime RT PreMix Kit (iNtRON Biotechnology, Inc, Korea) in which template RNA equivalent to 0.1 µgand distilled water were added into the Maxime RT PreMix tubes (containing Random

primer) to a total volume of 20µl. cDNA synthesis reaction was performed as follows; cDNA synthesis at 45° C for 60 min and RTase inactivation step at 95 °C for 5 min, using PCR Thermal cycler(Biometra, Germany).

Amplification and Quantification of cDNA

Complementary DNA was quantified with SYBR green real-time PCR using superReal premix plus kit (TIANGEN, China). A pair of primers specific for and internal to the efflux pump gene *PA1874* gene (5′-GCGTCGGCATCGATACCAAT-3′ [forward]) and (5′-ACGATCACC GTCACCGTCTC-3′ [reverse]) was used to amplify cDNA corresponding to mRNA as a measure of*PA1874* gene expression. For a control, the mRNA of the constitutively expressed*rpoD*gene was amplified and quantified with the primers (5′-GATCCGGAACAGGTGGAAGAC-3′ [forward]) and (5′-TCAGCAGTTCC ACGGTACCC-3′ [reverse]) (Zhang and Mah, 2008).

Each 25 µl quantitative real-time PCR mixture contained 5 µl cDNA (1µg cDNA), 12.5 µl Super RealPreMix Plus, 0.5 µl Rox dye and 0.3µM of each primer. The cycler was programmed as following; 15 min at 95° C, 40 cycles each with 10 sec. at 95°C, 25 sec. at 60°Cand 30 sec. at 72° C.

Statistical Analysis

Data were checked, entered and analyzed using SPSS version 19 for data processing and statistic. The variability of results were checked using Chi- square test (X2), Kruskall-Wallis test,Correlation co-efficient (r), P value equal or less than 0.05 was considered significant.

Results and Discussion

During this study, 186 different clinical samples were obtained from 186 hospitalized patients including 102 males and 84 females with their ages ranged from 1-69 years.Sixty four (34.4%) *P.aeruginosa* isolates were isolated from different clinical samples including 20 (36.3%) isolates from 55 infected wound exudate specimens, 15 (32.6%) from 46 burn exudate specimens, 18 (36%) isolates from 50 urine samples from catheterized patients, 11 (31.4 %) from 35 ETA of ventilated patients. There was no statistically significant difference (P>0.05) between them.

Twenty six (40.6%) *P. aeruginosa* isolates were biofilm +ve. There was statistically significant difference(P<0.05)in the distribution of biofilm +ve *P. aeruginosa* isolates between different clinical specimens (table 1).The highest percentage of biofilm +ve *P.aeruginosa* isolates(63.6%) is among ETA specimens and urine samples (P.<0.05).

Among different risk factors associated with patients, instrumentation for more than 8 days has increased the risk for biofilm formation than in instrumented patients with less than 8 days (P<0.05, Table 2).

The biofilm cells demonstrated higher resistance than counterpart planktonic cells to different antibiotics where the mean and median of biofilm +ve MBIC for ciprofloxacin; tobramycin and gentamycin were higher than those of MIC of their planktonic counterparts and more higher than those of biofilm –ve MIC. This differences were of statistically significant (P<0.05, Table 3) regarding the three antibiotics. SO biofilm form showed the highest degree of resistance as all of them (26 isolates)were resistant to all used antibiotics while 19 (73.1%) of the

planktonic counterpart showed resistant to ciprofloxacin, 16(61.5%) resistant to each tobramycin and gentamycin. On the other hand the biofilm –ve isolates shows 19(50.5%) of them resistant to ciprofloxacin, 24(63.2%) resistant to tobramycin and 25(65.8%) resistant to gentamycin, with high statistically significant difference between the groups($P<0.001$, Table 4).

The highest degree of expression of efflux pump gene *PA1874* was found among biofilm +ve *P. aeruginosa* isolates in biofilm form, lower degree was in their planktonic counterparts and the lowest degree of expression was among biofilm –ve isolates. The difference between three groups wasof statistically significant ($P<0.05$, Table 5, Fig 1).There was strong +ve correlations between biofilm –ve MIC, planktonic biofilm +ve MIC and biofilm +ve MBIC of ciprofloxacin and tobramycin and expression of efflux pump gene *PA1874* with statistically significant differences (Table 6).

P. aeruginosa is an opportunistic human pathogen, its infection is life-threatening for immuno-compromised patients and is rich in quorum sensing molecules and virulence factors and has the ability to form a biofilm, which is thought to contribute to its adaptability and versatile lifestyle. Furthermore, *P. aeruginosa*is often resistant to treatment and develops antibiotic resistance over time. Biofilm-associated growth is thought to play a key role in bacterial adaptability and antibiotic resistance **(**Sigurdsson *et al.,* 2012)

In this work, 64 (34.4%) *P.aeruginosa* strains were isolated from 186 different clinical specimens as was reported previously (Ashour *et al.*,2003). In our study, *P. aeruginosa* was detected in 20(36.3%) of 55 wound exudates. This is almost in agreement with a study made in an Italian hospital (Cestari *et al.*,1999) and with an Egyptian study (Ashour *et al.,* 2003).
In contrast, lower percentages were obtained by other investigators as Begum *et al.* (2013) isolated it from 9.5% of wound exudates.

In the current work, *P. aeruginosa*was recovered from 15(32.6%) of 46 burn exudates. This result is in agreement withAshour *et al.* (2003) and in contrast with Nasser *et al.* (2003) who isolated it only from 21.6% of burn wound infection in Ain Shams University Hospitals. Also,*P. aeruginosa*was isolated from 18(36%) out of 50 urine samples as was reported by Vaziri *et al.* (2011). However, lower percentages were obtained by several other investigators (Ashour *et al.*,2003;Begum *et al.*,2013)

Concerning chest infection in artificially ventilated patients, *P. aeruginosa* was recovered from 11 (31.4 %) out of 35 cases. This results is near to that obtained by Medell *et al.* (2013), however, a lower percentage (15.3%) was reported by Ezzat *et al*.(2002). The discrepancy in the percentages of *P. aeruginosa* isolated in our study and other studies could be attributed to the difference in hospital environment, clinical conditions of patients in different countries and numbers of clinical specimens investigated in different studies

Out of the 64 *P.aeruginosa* isolates, 26(40.6%) were biofilm +ve by tube method. This result is in consistence with Hassan *et al.* (2011). On the other hand, higher percentage was detected by Gupta *et al.* (2011) who found that all of *P.aeruginosa* isolates were biofilm producers. In spite of Mikucionyte *et al.* (2014) who did not find any statistically significant differences in biofilm production among the *P. aeruginosa* strains recovered from different sources, in our work, there

was statistically significant difference in the distribution of biofilm +ve and biofilm –ve *P. aeruginosa* isolates in relation to different clinical specimens. The highest significant percentage of biofilm +ve isolates was from ETAs (63%) followed by urine samples of catheterized patients (55.6%). These results are in consistent with previous several studies (Gil-Perotin *et al.,* 2012; Hisham *et al.*,2012; Sumithra *et al.*,2014).

Concerning the risk factors associated with biofilm +ve *P. Aeruginosa* infection, only instrumentation either by urinary catheter or endotracheal tube for >8days considered as risk factor as it increased the risk for acquiring biofilm 4 times than in instrumented patients <8 days. This agrees with Donlan (2001) who found that the longer the urinary catheter remains in place, the greater the tendency of organisms to develop biofilms, as only 10% to 50% of patients undergoing short-term urinary catheterization (7 days) but virtually all patients undergoing long-term catheterization (>28 days) became infected with biofilm +ve infection. Also, Ockmore and Feneley (1996) found that about 50% of the patients who undergo long term urinary catheterization (> 2weeks) will suffer the complication of catheter blockage by bacterial biofilms.

This results can be explained by the fact that microorganisms must adhere to the exposed surfaces of the device long enough to become irreversibly attached. Once these cells irreversibly attached it can produce extracellular polysaccharides to develop a biofilm (Donlan,2001).

Empirical treatment of most of hospitalized patient in Zagazig University Hospitals with gentamycin, tobramycin and ciprofloxacin in different types of infection without culture and sensitivity tests could increase resistance to these antibiotics themselves and to other groups of antibiotics and make selective pressure to resistant strains, especially when this resistance can be transferred on transferable genes as resistance to aminoglycoside by modifying enzymes.

We studied the antibiotic susceptibility of *P.aeruginosa* isolates to ciprofloxacin, tobramycin and gentamycin by broth microdilution method and we found that of the biofilm –ve isolates, 50% were resistant to ciprofloxacin, 63.2% were resistant to tobramycin and 65.8% were resistant to gentamycin. On the other hand, much lower percentages were detected by different studies in other countries (Lambert, 2002;Brown and Izundu, 2004; Lister *et al.*,2009)

In the same direction,biofilm cells demonstrated higher resistance than planktonic cells to the same antibiotics. The concentration of antibiotics required to inhibit biofilm bacteria (MBIC) was greater than those required to inhibit planktonic counterparts (MIC) and according to NCCLS (2007)guidelines, all of biofilm cells (26 isolates) were resistant to all used antibiotics while 19 (73.1%) of the planktonic counterpart showed resistant to ciprofloxacin, 16(61.5%) resistant to each tobramycin and gentamycin.. These results are more or less in agreement with previous studies(Agarwal, 2005; Abbas *et al.*,2012 ; Sepandj *et al.*,2004).

When the expression of efflux pump gene *PA1874* was measured, it was higher among biofilm +ve*P. Aeruginosa* isolates in biofilm form, lower degree is in their planktonic counterparts and the lowest degree of expression was among biofilm –ve isolates. These results are consistence with the results

reported by Zhang and Mah (2008) who found that *PA1874* gene was 10-fold more highly expressed in biofilm cells than in planktonic cells, providing an explanation for why these genes are important for biofilm more than planktonic resistance to antibiotics, they suggested that *PA1874-1877* is acting like a multidrug efflux pump rather than a specific transporter for one antibiotic or one class of antibiotics affecting sensitivity to tobramycin, gentamycin and ciprofloxacin. In other study, Zhang *et al.* (2013) found that other three operons *PA0756-0757*, *PA2070* and *PA5033* were similarly regulated, and quantitative real-time PCR of these genes exhibited a higher level of gene expression in biofilm cells, suggesting that these genes are important also for biofilm resistance to the same three antibiotics.

Table.1 Distribution of Biofilm +ve and Biofilm –ve *P.aeruginosa* Isolates in each Clinical Specimen

Source of clinical specimen	No. of samples No.=64	biofilm-ve (No.=38)		biofilm +ve (No.=26)	
		No.	%	No.	%
Wound	20	16	80.0	4	20.0
Urine	18	8	44.4	10	55.6
ETA	11	4	36.4	7	63.6
Burn	15	10	66.7	5	33.3
X2	7.94				
p.value	0.047*				

X^2= chi square test *Significant p <0.05
ETA: endotracheal tube aspirate

Table.2 Risk Factors Associated with Biofilm +ve*P.aeruginosa* Isolates

Risk factor	Biofilm -ve (No.=38)		Biofilm +ve (No.=26)		Relative risk (RR) (95%CI)	X^2	P-value
	No.	%	No.	%			
Age							
<40	22	68.8	10	31.2	2.2	2.33	0.127
≥40	16	50.0	16	50.0	(0.71-6.95)		
Sex							
Female	17	63.0	10	37.0	1.3	0.25	0.618
Male	21	56.8	16	43.2	(0.42-4.06)		
Duration of hospitalization							
<7 days	22	52.4	20	47.6	0.41	2.48	0.115
≥7 days	16	72.7	6	27.3	(0.12-1.42)		
Duration of instrumentations.	12	63.2	7	36.8	4	4.31	0.038*
<8 days	6	30.0	14	70.0	(0.88-19.32)		
≥8 days							
Diabetes mellitus	15	50.0	15	50.0	0.48 (0.15-1.48)	2.06	0.151

X^2= chi square tes *Significant p<0.05

Table.3 Comparison Between Biofilm –ve MIC, Planktonic Biofilm +ve MIC and Biofilm +ve MBIC of Ciprofloxacin, Tobramycin and Gentamycin (µg/ml)

Antibiotic		Biofilm-ve MIC	Planktonic biofilm +ve MIC	Biofilm+ve MBIC	KW	P-value
Ciprofloxacin	$\overline{X}$ ± SD	0.5±0.3	20.7±12.4	62±34.9	10.355	0.006*
	Median	0.375	12	48		
Tobramycin	$\overline{X}$ ± SD	11.8±5.6	82.8±46.1	288±188.8	10.44	0.005*
	Median	1.5	48	265		
Gentamycin	$\overline{X}$ ± SD	24.8±15.6	116.2±99.5	429.3±202.5	9.069	0.011*
	Median	5	24	512		

KW=Kruskall Wallis test *Significant p <0.05

Table.4 Patterns of Antibiotic Resistance of Bifilm –ve, Biofilm +ve (planktonic) and Biofilm +ve (biofilm) of *P.aeruginosa* Isolates

Antibiotics	Biofilm-ve		Biofilm +ve (planktonuc)		Biofilm +ve (biofilm)		X^2	P-value
	Susceptible No.&%	Resistant No.&%	Susceptible No.&%	Resistant No.&%	Susceptible No.&%	Resistant No.&%		
Ciprofolxacin	19(50.0)	19(50.0)	7(26.9)	19(73.1)	0(0.0)	26(100.0)	63.08	<0.001**
Tobramycin	14(36.8)	24(63.2)	10(38.5)	16(61.5)	0(0.0)	26(100.0)	60.03	<0.001**
Gentamycin	13(34.2)	25(65.8)	10(38.5)	16(61.5)	0(0.0)	26(100.0)	62.35	<0.001**

X^2= chi square test **High significant p<0.001

Table.5 cDNA Concentration of *PA 1874* Efflux Pump Gene in Biofilm –ve, Biofilm +ve (planktonic) and Biofilm +ve (biofilm)

c DNA concentration	Biofilm-ve	Biofilm +ve (Planktonic)	Biofilm+ve (Biofilm)	KW	P-value
$\overline{X}$ ± SD	0.001±**0.001**	0.9±**1**	2.4±**1.8**	11.942	0.00*
Median	0.00108	0.5317	1.8247		

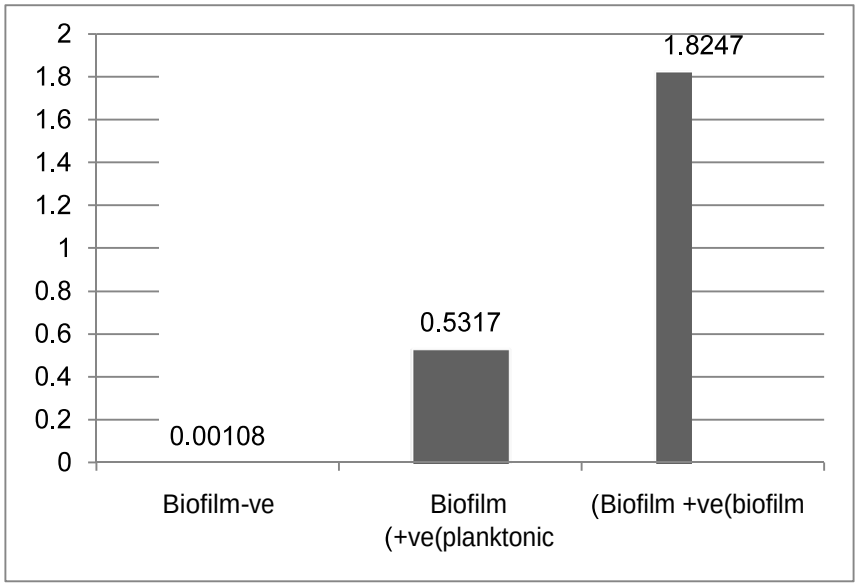
KW=KruskallWallis test *Significant P<0.05Pseudomonas

Table.6 Correlation Between Biofilm -ve MIC, Planktonic Biofilm+ve MIC and Biofilm +ve MBIC (µg/ml) and cDNA Concentration of *PA 1874* Efflux Pump Gene

c DNA concentration	Biofilm -ve MIC		Planktonic biofilm +ve MIC		Biofilm +ve MBIC	
	(r)	p.value	(r)	p.value	(r)	p.value
Ciorofolxacin	0.989	<0.001**	0.931	0.007*	0.965	0.002*
Tobramycin	0.878	0.021*	0.931	0.007*	0.977	0.001*
Gentamycin	0.898	0.015*	0.814	0.049*	0.645	0.167

*Significant *P*<0.05 **High significant *P*<0.001

Figure.1 cDNA Concentration of *PA 1874* Efflux Pump Gene in Biofilm –ve, Biofilm +ve (planktonic) and Biofilm +ve (biofilm)



From this Study We Can Conclude that

P.aeruginosa is an important nosocomial pathogen and has ability to form biofilm that may be the main cause of multidrug resistance among *P.aeruginosa*.

Efflux pump*PA1874* is an important mechanism of resistance in *P. aeruginosa* biofilms that showed the highest degree of expression in biofilm form.

Increased MIC of biofilm –ve and planktonic biofilm +ve and MBIC of biofilm +ve*P. aeruginosa*isolates of ciprofloxacin, tobramycin and gentamycin were associated with increased expression of this novel efflux pump gene *PA1874*.

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