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ANTIBIOGRAM STUDY AND PREVALENCE OF *PSLÁ* GENE AMONG BIOFILM *PSEUDOMONAS AERUGINOSA* PRODUCERS ISOLATED FROM SOME CLINICAL SPECIMENS IN THI_QAR PROVINCE

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ABSTRACT

One of the major causes of hospital-acquired contagion due to the rising antibacterial resistance is opportunistic *Pseudomonas aeruginosa*. Biofilm formation is an important virulence factor increase the pathogenicity infectious of *P. aeruginosa*, since sessile bacteria are protected in an extracellular matrix of a polysaccharide. The expression of expolysaccharide (EPS) manufacturing locus (*pslÁ* gene) can be significant for biofilm formalization by *P. aeruginosa*. The goal of this research estimate antibiotics resistance pattern and apportionment of the *pslÁ* gene among biofilm construction *P. aeruginosa* isolates collected from different infections sources.

There were 113 infection samples collected as swabs and sputum from burns, post-surgical wounds, ear (otitis media), and respiratory tract. They cultured on different selective and differentiation media, next to *P. aeruginosa* identification by traditional bacteriological, API 20E strip, and molecular PCR technique by 16S rRNA. The antibiogram of isolates achieved by exposed to ten antibiotic items with disk diffusion method according to standard protocol of Clinical and Laboratory Standards Institute. Biofilm formation was detected qualitatively and quantitatively by 96-well microtiter plate test (MPT). Genotypic prevalence of *pslÁ* gene among the isolates performed via monoplex thermocycler of PCR technique.

Of 113 different infection specimens there were 109 gave positive growth. *Pseudomonas aeruginosa* isolates were allocated to; 13/31 (35.5 %) burns, 8/26 (30.7 %) wounds, 11/29 (34.5 %) otitis, and 7/23 (30.4 %) sputum. Of 39 there was 23 multidrug resistance (MDR), 5 pandrug resistance (PDR) and the rest multidrug sensitive (MDS). The highest resistance was against Ticarcillin, Netilmicin, Piperacillin, and Ticarcillin/clavulanic acid while Amikacin, Meropenem, and Ciperofloxacin more effective with all isolates except burn isolates. The quantitatively of biofilm formation by MPT appeared 17 (43.6 %), 9(23.1 %), 7 (18.0 %), and 6 (15.4 %) were strong, moderate, weak, and non- biofilm producers. The prevalence of biofilm *pslÁ* gene was in 35 (90.0 %) isolates. All strong biofilm and 4 non-biofilm producers harbored target gene, while 1 moderate, 1 weak, and 2 non- biofilm producers not harboring *pslÁ*. All PDR and most MDR isolates were strong biofilm formation.

P. aeruginosa is still developing the resistance to most antibiotics especially with strong biofilm formation isolates mostly of *pslÁ*gene harboring which increasing the concern and difficulty of bacterial infections treatment. Therefore, the need to find new strategies for healing is urgent.

Keywords: *Pseudomonas earuginosa*, antibiogram, MDR, PDR, biofilm, *pslÁ*

INTRODUCTION

One of an important opportunistic for human pathogen is *Pseudomonas aeruginosa* a gram-negative bacterium which causes diversified life-threatening hospitalist infections in patients of immunocompromised and sufferers from burns, post-surgical wounds, otitis media and preponderant pathogen in cystic fibrosis (Overhage *et al*., 2005). Also, Beyond its naturalistic resistance to numerous medication, *P. aeruginosa* capable to produce slime and biofilm, a complex biological system, makes the rescue by immune protection systems and antibio-curative (Rasamiravaka *et al*., 2015). *P. aeruginosa* has been a paradigm organism for biofilm formation studies and the biofilm matrix structure is polysaccharides elements, as they contribute to the gross biofilm architecture and to the reluctance of biofilm- grown bacteria against appointed antimicrobial agents (Ghafoor *et al*., 2011). So biofilm became a huge dilemma that interest of researchers in the trend towards elimination, such as the Iraqi studies mediated by Al-Mousawi and Al-Kabbi, (2017) used hot an aqueous extract of leaves *Ziziphus spina Christi* (Sidr) with 50mg/ml investigated, the results revealed a high synergism effect between moxifloxacin, Penicillin and plant extract to inhibit biofilm formation of clinical *Staphylococcus arueus*. There are three exopolysaccharides have been appeared to be involved in biofilm production by *P. aeruginosa* including alginate, Psl, and Pel (Franklin *et al*., 2011). Psl is a mannose- plentiful polymer with an essential role in the prime stages of biofilm formation by *P. aeruginosa* as well as in its stability. Psl build in a helical structure around these cells which increase the cell-to-surface and

cell-to-cell interaction indispensable for biofilm construction (Ma *et al*., 2006). Manufacturing of Psl is intervene by the *psl* gene cluster(*pslÁ-pslÓ*) and the first gene of this operon is *pslÁ*2231 which is most significant gene critical for Psl synthesis (Overhage *et al*., 2005, Ghafoor *et al*., 2011). The goal of this study was empirical investigation for biofilm producers and prevalence of *pslÁ* gene in multidrug resistance *P. aeruginosa* isolated from Thi_Qar province patients.

MATERIALS AND METHODS

Assembling of specimens: Samples gathered from patients suffering from burns, wounds, respiratory, and otitis media infections in different hospitals of Thi_Qar province, during beginning of April until the terminus of September/2017, using two disposable sterile cotton swaps each specimen. There were 113 specimens from burns; wounds, ear, and sputum are 32, 27, 29 and 25 respectively. Patients included both male and female, different ages, diverse local regions and together urban and rustic habitat. According to Murray *et al*. (2007), the transportation of all specimens were immediately transferred to The Microbiology Laboratory - College of Science/ Thi_Qar University, for cultivating, identification and other tests.

***P. aeruginosa* isolation and identification:** Based on Gillespie and Hawkey (2006), the cultivating on rich, differentiation and selective media, included, 5% Blood sheep agar, Nutrient agar, MacConkey agar, Cetrimide agar, King agar, in conjunction with traditional biochemical tests comprised; gram staining, oxidase, catalase, triple sugar iron agar(TSI), oxidation/ fermentation glucose, growth and non-growth overnight at 42°C and 4° C respectively were performed to characte-rize *P. aeruginosa* colony morphologically, cell microscopically and metabolically. By analytical Profile Index (API) 20E Kit (BioMerieux, France) microtubules enzymatically tests to support strains identification. Finally, the molecular detection using 16S rRNA boosted by conventional PCR technique standard on Persing *et al*., (2016), for obtaining pure isolates diagnostic at a species level, which stored in Brain Heart Infusion (BHI) broth; slant with agar and broth contain 30 % glycerol at refrigerator temperature and -20°C respectively for next experiments.

Antibiogram test: The disk diffusion susceptibi lity method for antibiogram testing (Kirby- Bauer method) was performed according to guidelines of Clinical Labrotary Standard Institute (CL-SI), *NCCLS*, (2012).

There were ten patterns of Antibiotic discs (Bioanalyse–Turkey) used in this study included Ciprofloxacin (CIP-5µg/disk), Amikacin (AK-30µg/ disk), Netilmicin (NET-30 µg/disk), Ceftazidime (CAZ-30µg/disk), Cefepime (FEP-30µg/disk), Mer - openem (MEM-10µg/ disk), Piperacillin (PR- L-100µg/disk), Aztreonam (AZT-30µg/disk), Ticarcillin (TIC-75µg/disk) and Ticarcillin/Clavulanic acid (TCC-75/10 µg/ disk). Identical pure colonies of *P. aeruginosa* was presubcultured on nutrient agar (N.A) plates at37°C, resuspended the bacterial suspension was adjusted to the turbidity at a McFarland standard no. 0.5 (1.5×10^8) CFU/ ml. The dried surface of Muller Hinton Agar (MHA) plate inoculated by streaking the swab over the entire sterile agar sur-face after ensured an even distribution of inoculums. The antibiotic test disks were applied firmly on the agar surface within 15 min of inoculation of the plates. After an overnight incubation at 37°C, zones of inhibition were measured and compared to the CLSI guidelines CLSI (2012). Multidrug resistance (MDR) was defined as resistance to at least one item from every different three antibiotic categories and pandrugs resistance (PDR) was resist to all antibiotic tested as described by Magiorakos *et al*., (2011).

Quantitative biofilm formation assay: Quantitative biofilm production was determined using a semi-quantitative in vitro adherence using 96-well flat-bottomed polystyrene tissue culture plate (Costar2797/ Serocluster™-USA) assay as an indicator of biofilm formation based on Stephanovic *et al*., (2007). In short; adequate pure identical colonies, grown overnight on Tryptic soy agar (TSA) were suspended in a tryptic soy broth (TSB) supplemented with 4% glucose, diluted 1:100. After that, aliquots of (0.1ml per-well) of each isolate suspension were then inoculated into eight- wells and wells containing TSB media (without bacteria) served as a negative control. Incubated overnight at 37°C. The contents of the wells discarded and well washed three times with 200µL of phosphate-buffered saline (pH 7.2). Subsequent, the plates should be drain. Then, fixation with 160 µL of 99 % methanol for 20min. The microtiter plates left to air dry overnight in an inverted position of Sequent. The adherent biofilm layer formed in each well was stained with 165µl of 1 % crystal violet tincture, for ¼ hour at room temperature. Later, washing and drying plate. The dye bound to the adherent cells was dissolved by adding 180µL of ethanol 96 % per well. Finally, the optical density (OD) of each well stained with crystal violet is measured at 570 nm using micro ELISA autoreader (BioTek, ELx800-UK). The experiment was repeated three times separately for each strain and the average values were calculated with standard deviation (SD). The interpretation of biofilm pro-

duction was done according to the criteria of Stepanović *et al*.,(2000) as in Table 1.

Table 1: Classification strength scope of biofilm formation.

OD value	Biofilm ranking
OD≤ ODc*	Non
ODc< OD≤ 2×ODc	Weakly
2× ODc<OD≤ 4×ODc	Moderate
4× ODc<OD	Strong

* ODc: The cut-off OD= 3x standard deviations above the mean OD of the negative control

***P. aeruginosa* DNA extraction:** Aliquot of 1.5 ml overnight reactivated growth in LB broth was added in 1.5 ml epndroff tube and centrifuged at 12,000×g for 5 min. Then, removed the supernatant while the remaining pellet employed for extraction and purified total *P. aeruginosa* DNA based on manufacturer`s instructions recommended, by used commercial DNA extraction kit via Quick Bacteria Genomic DNA Extraction Kit (Dongsheng Biotech, China). After that, the final purified DNA quantitatively subjected to determine both concentration by (ng per µl) and purity by the ratio of dividing 260/280nm readings were measured via Nano Drop spectrophotometer device (Avans Biotechnology, TAIWAN). Then, quantitatively detection of total bacterial DNA was performed by 1% agarose gel stained with ethidium bromide using gel electrophoresis system (Bioneer-Korea) at 100V for ¼ h. Later, illuminated under UV- transilluminator spectrophotometer Desktop Gel Imager (Optima, Japan) according to Sambrook and Russel *et al*., (2001). Finally, the genomic DNA was conserved at -20°C for future analyzes.

PCR technique for detection 16S rRNA and *pslÁ* genes: The reaction mixture of PCR was 25 µL in a total volume containing; 12.5µL of premixed ready mastermix which mixed with forward and reverse primers (10 pmol/1µl) each, the specific primers of genes provide from *AlphaDNA* (Montreal, Canada) and their sequence listed in Table (2), 1 µL of extracted genomic DNA, and 9.5 µL of nuclease-free double distilled water.

Table 2: List primers for PCR detection of the biofilm *pslA* and *16S rRNA* associated genes used in this study.

Primer	PCR Sequence (5'-3')	Annealing temp (°C)	Goal	Amplicon size (bp)	Position*
PA-SS-F *PA-SS*-R	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	58	*P. aeruginosa detection*	956	189-206 1124-1144
pslÁ -F1 *pslÁ* -R1	CACTGGACGTCTACTCCGACGATAT GTTTCTTGATCTTGTGCAGGGTGTC	55	Psl Expolysacchride	1119	1-25 1095-1119

Amplifications were carried out in Gradient Veriti ™ 96-Well Thermal Cycler (Applied Biosystems, Singapore) thermo-controller. A thermal step program was used including the following parameters: firstly; for16S rRNA (1535 bp) amplification was used to diagnostic *Pseudomonas*, as *aeruginosa* species cited from Spilker *et al*., (2004). The reaction program was illustrated in Table (3). Secondly; biofilm production *pslÁ* (1437bp) gene, that thermal protocol amplified based upon Hou *et al*., (2012) demonstrated in Table (4). Both, amplified products were held at 4°C until analysis. Amplification products were analyzed using 1.7 % (w/v) agarose gel (promega-Korea) stained with ethidium bromide using 100 bp DNA ladder (Dongsheng Biotech, China) as reference guide in TBE electrophoresis, and visualized specific bands by illuminated under UV-Spectrophotometer image analysis system according to Sambrook and Russel (2001)

Table 3: PCR program and parameters amplification of *16S rRNA*gene.

No.	Steps		Temperature (°C)	Period	
1	Initial denaturization		95	2 min	
2	a	Denaturation	94	20c se	25 Cycles
	b	Annealing	58	20 sec	
	c	Extension	72	40 sec	
3	Final extension		72	1 min	

Table 4: PCR program and parameters amplification of biofilm *pslÁ*gene.

No.	Steps		Temperature (°C)	Period	
1	Initial denaturization		94	10 min	
2	a	Denaturation	94	30c se	30 Cycles
	b	Annealing	55	30 sec	
	c	Extension	72	1 min	
3	Final extension		72	10 min	

Statistical Analysis: All experiments were performed at least in duplicate or triplicate, The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters. Chi-square test (χ^2) was used to significant compare between averages and percentages number isolates of values are recorded in the tables and figures.

RESULTS AND DISCUSSION

Isolation and identification of *P.aeruginosa* by traditional methods: The common microbiological detection methods appeared that of 113 clinical specimens, there were just four given no growth, while 109 manifested positive culture, of this total there were 41 indicated as *Pseudomonas aeruginosa* , the results of media growth and biochemical tests listed in Table 5.

Table 5: Results of traditional microbiological detection methods *P. aeruginosa*.

No.	Media and test	Result
1	Yellow on MacConkey agar	+
2	Lactose fermentation	-
3	Pyocinine on nutrient agar	+
4	Gram stain	-
5	Growth on Cetrimide agar at 44°C	+
6	Yellow greenish color on King A agar	+
7	Catalase	+
8	oxidase	+
9	Triple Sugar Iron slant	**Alk / Alk**
10	glucose oxidation	+
11	Growth at 42°C	+
12	No growth at 4°C	_

For more accurate detection, the strip of API 20E metabolically and enzymatically assay. The results revealed that of 109 there were 39 (35.8 %) specimens restricted with *P. aeruginosa*. Finally, the genotypic analysis via PCR reaction with specific *16S ribosomal RNA* (956 pb) primer Figure 1, finishing the confused about diagnosis and confirm the API 20E test proved that 39 specimens were the accurate conclusive outcome and within *Pseudomonas aeruginosa* distributed at 13/31 35.5 %) burns, 8/26 (30.7%) wounds, 11/29 (34.5 %) otitis, and 7/23 (30.4 %) sputum. there were no significant differences in percentages number isolates among isolating sources (Table 6).

Table 6: Percentages specimens and number isolates from different isolating sources.

Specimen sources	No. and % of Specimens	No. and % of isolates
Burns	31(28.4%)	13 (35.5%)
Wounds	26(23.9%)	8 (30.7%)
Respiratory Sputum	23(21.1%)	7 (20.4%)
Ear swabs	29(26.6%)	11 (34.5%)
Total	109(100.0%)	39 (35.78%)
Chi-Square(χ^2)	2.124 NS *	1.952 NS
*** (P<0.05), NS: Non-Significant.**		

The result of specimens in recent search agreed in diagnostic but highest in percentage and number with Iraqi results of AL-Shamaa *et al*., (2016) that their isolated was just from burn infections showed of 111 there were 25 (22.5 %) *P. aeruginosa*. This difference may be due to variation of isolating sources of present study. No doubt always the common bacteriological methods for detection not enough at level species and must be supported with modern automated tests like API 20E strip assay and for increasing precisely detected the molecular technique is useful and perfect suggested by Jaturapahu *et al.*, (2005).

***P. aeruginosa* DNA extraction:** Determination of DNA concentration and purity were done using the Nanodrop device previously mentioned. The average DNA concentrations of the extracts were 288.2 ng/µl and the purity was ranging 1.73-1.87.

Molecular detection by 16S rRNA gene: Just 2 isolates didn't match with 16S rRNA primer, that 39 isolates were belong *P. aeruginosa* accurately, showed in Figure 1, genotypic detection is a precisely and favorable for bacterial diagnosis at species level other than traditional technique especially with *P. aeruginosa* confirmed from Spilker *et al*., (2004).

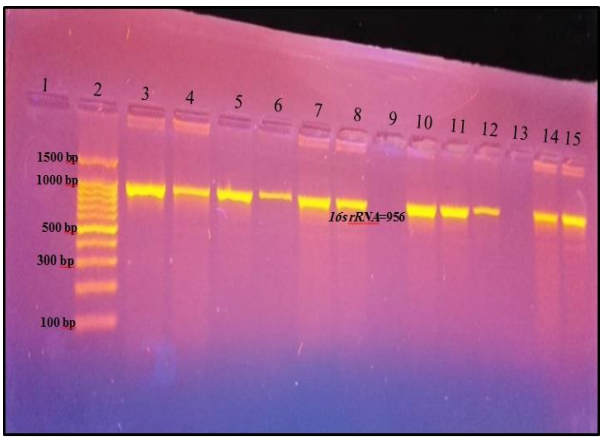
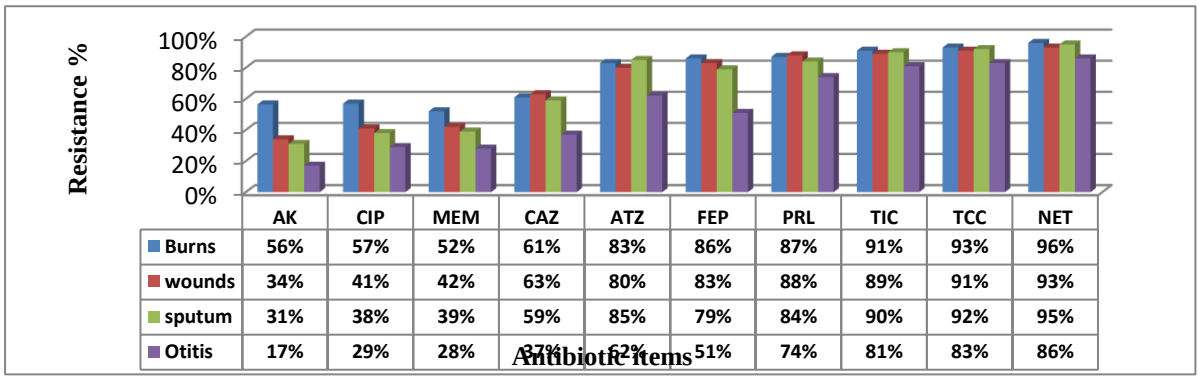


Figure 1: Gel electrophoresis of amplified PCR product of 16S rRNA gene (956 bp) in monoplex PCR at 70v for 90 min in 1.7 % agarose, TBE (1x), stained with ethidium bromide. Lane (2): DNA ladder (100bp), lane (1) negative control (without DNA), all the lanes were positive for the target gene except the lanes from (9-13) were negative result.

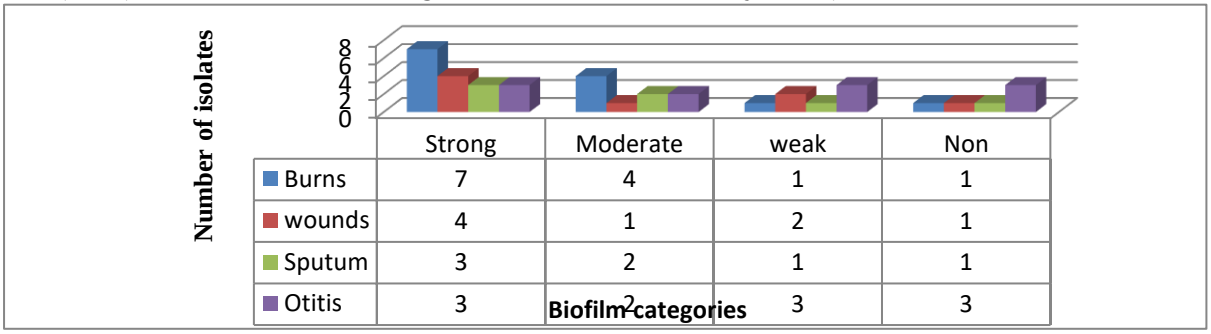
Antibiogram assay: Of 10 antibiotic items *P. aeruginosa* exhibited diversity in resistance phenomena, the isolates were a highest rate of resistance against, Piperacillin, Ticarcillin, Ticarcillin/ Clavulanic acid, and Netilmicin and there was no significant difference among both these antibiotics (χ**2**=0.424**)** and sources of isolation (χ**2**=0.235**)** in resistance percentages at p<0.01. The rate impedance was high with Ceftazidime, Aztreonam, and Cefepime. The resistance percentage against Amikacin, Ciprofloxacin, and Meropenem less and they were most effective with all isolates excepted burns isolates showed highly significant difference (χ**2**=9.466**)** at p<0.01 reluctance versus all antibiotics, Figure (2). The results of antibiogram exhibited 23/39 (59 %) were Multi-DR of these 5/23 (217 %) were resistant to all the tested agents (pan-drug resistance/ PDR). This resistance is developing to Multi-DR phenomenal type in *P. aeruginosa* suggested by Hirsch and Tam, (2011) due to diversified mechanisms including outer membrane proteins (porins) loss, beta-lactamase production, multidrug efflux systems, and targets modifications. The Iraqi study by Al-Ammar and Al Maghathry (2017) showed the *Acenitobacter bumannii* is another multi-DR bacteria against variety antibiotic classes which increasing the treatment concern difficulty of nosocomial infections besides *P. areuginosa*. Also, it was demonstrated that local isolates of burn infections exhibited obvious resistance to Imipenem and Meropenem (Ghaima *et al*., 2016).



	AK	CIP	MEM	CAZ	ATZ	FEP	PRL	TIC	TCC	NET
Burns	56%	57%	52%	61%	83%	86%	87%	91%	93%	96%
wounds	34%	41%	42%	63%	80%	83%	88%	89%	91%	93%
sputum	31%	38%	39%	59%	85%	79%	84%	90%	92%	95%
Otitis	17%	29%	28%	37%	62%	51%	74%	81%	83%	86%

Figure 2: The percentage resistance of antibiogram assay for *P. aeruginosa* isolates against 10 antibiotic items.

Quantitative measurement of biofilm assemblage: Quantitative biofilm determination using the microtiter plate assay revealed that 33/39 (84.6 %) isolates produced biofilm in different strength levels and the remaining 6 isolates were non-biofilm producers (Figure 3). The burn isolates were more with strong biofilm7/13 (54.0 %) with adequate to be significantly than other strains, he large number of total isolates were strong (OD> 0.653) and moderate (0.327<OD≤0.653) biofilm grade and the weakly (0.158<OD≤0.317) and non- biofilm (OD≤0.163) producers were more with otitis (ODc=0.158, data did not show). Although this results in this study less than in dominant percentage of biofilm producers by Corehtash *et al*., (2015) was 92.4% of *P. aeruginosa* isolates that form the biofilm detected by the same test, but remained in higher percentage of biofilm formation than non-formation isolates. The current study compatible with what was found Aziz and Al-Jubori, (2017) the percentage of strong biofilm producers 57.3 % with same (OD≥0.67) of *Klebsiella pneumonia* isolated from Iraqi burn infections. The grade and strength of biofilm production depend upon on site and type of infection, period of bacterial colonization, activity of host immunity, extracellular polymeric substances (EPS) composition and environmental adaptation, therefore; there are diversity in bacterial capability of biofilm formation not just among genus but among the same species too (Hall-Stoodley and Stoodley, 2009).



	Strong	Moderate	weak	Non
Burns	7	4	1	1
wounds	4	1	2	1
Sputum	3	2	1	1
Otitis	3	2	3	3

Figure 3: Quantitative biofilm formation of *P. aeruginosa* isolates estimated by micro-titer plate assay.

PCR technique and genotypic detection of biofilm *pslÁ* gene: The amplification product of *pslÁ* gene for a number of *P. aeruginosa* isolates (1119 bp) was showed in Figure (4), there were 35/39 (90.0%) carriage the *psl Á* gene, one and two from both burns, wounds and otitis isolates respectively didn't carry this gene. The result of prevalence and harboring for *pslÁ* gene in *P. aeruginosa* iso-lates in recent work was highly percentage than Iranian studies of Emami *et al*., (2015) and Heydari and Eftekhar, (2015) were 42.9% and 52% respectively. In this research there was a relationship observed between antibiotic resistance profiles with both biofilm-positive and bio-film-negative isolates, by mean, biofilm producers have been more resistance to most antibiotics than non-producer strains, and all isolates showed as both PDR and MDR in antibiogram test were gave a very strong biofilm formation and own *pslÁ* gene (figure 5). According to Gaddy and Actis (2009) the multi-drug resistance correlates with the ability to form biofilm on abiotic and biologi- cal surfaces. The working by Drenkard Ausubel (2002) were found that the antibiotic-resistant variants of *P. aeruginosa* had high ability to form biofilm both in vivo and in vitro, which by Bolaji *et al*., (2011) explicated that the transferring of such high-resistance isolates can occur in environ-ments such as groundwater or somehow in health-care centers and clinical instruments where it becomes a potential risk for the human health. To evaluate the essential role of *pslÁ* in biofilm formation the study by Overhage *et al*., (2005) generated a non-polar isogenic *pslA* knockout mutant of *P. aeruginosa*. The observation of this mutant was impaired in attachment and biofilm formation and the mutant showed about 30% less attachment to tissue culture plates than the respective wild type. In another working by Ghafoor *et al*., (2011) found that *pslA* mutant was still able to form biofilm, but this biofilm was flat, fragile and much more compact than the biofilm formed by all other studied mutants, and both live and dead cells were present in this biofilm. From suggestion of Emami *et al*., (2015) emphasized that it seems the *pslÁ* gene had association with biofilm formation, since it was widely distributed among the biofilm-producing isolates. These results showed that the *pslÁ* gene was an important factor to form biofilm. However, since this gene was found in all the biofilm producers including strong and weaker isolates, perhaps there were other genes or factors that played role in forming biofilm, Therefore, it should be considering investigating other genetic and phenotypic factors for more future studies.

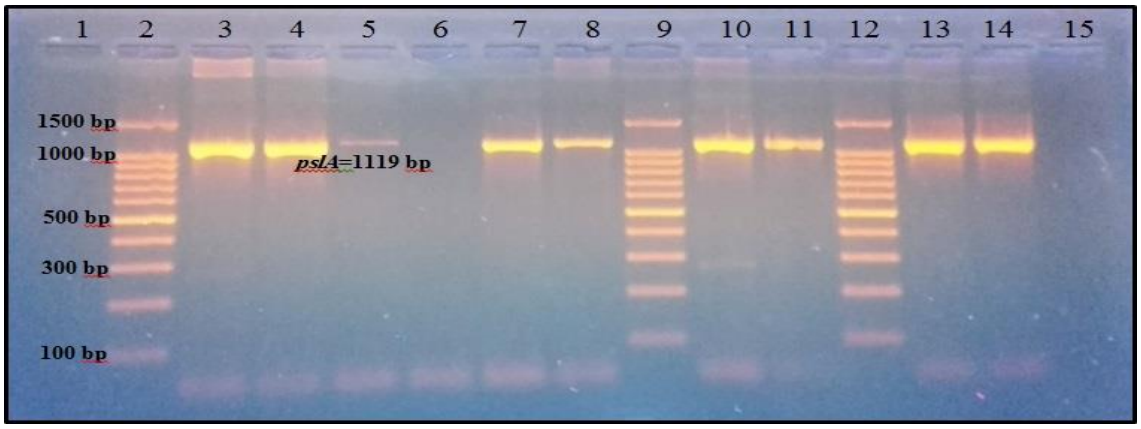
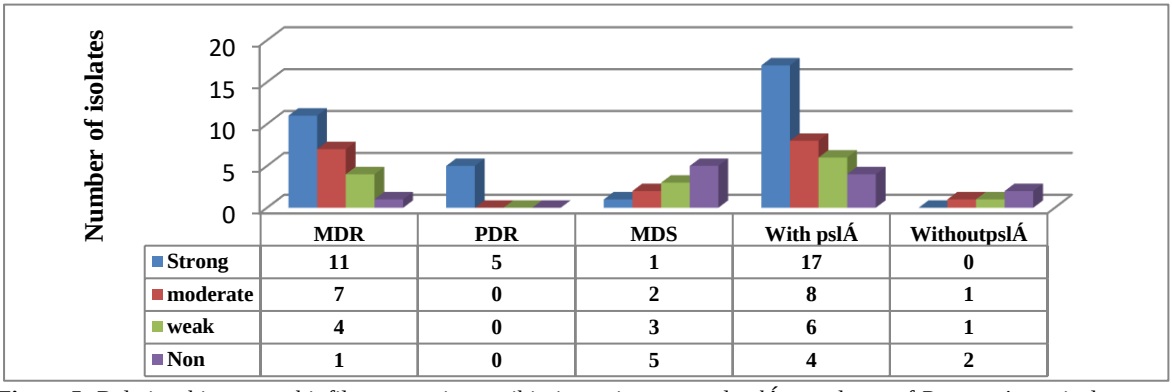


Figure 4: Gel electrophoresis of amplified PCR products of biofilm *pslA* gene (1119 bp) in monoplex PCR at70vfor 90 min in 1.7% agarose, TBE (1x), stained with ethidium bromide. Lane (2,9 and 12): DNA ladder (100bp), lane (1) negative control (without DNA), all the lanes were positive for the target gene except the lanes from (6-15) were negative results.



	MDR	PDR	MDS	With pslÁ	WithoutpslÁ
Strong	11	5	1	17	0
moderate	7	0	2	8	1
weak	4	0	3	6	1
Non	1	0	5	4	2

Figure 5: Relationship among biofilm categories, antibiotics resistance, and *pslÁ* prevalence of *P. aeruginosa* isolates.

CONCLUSION

In conclusion, biofilm production has been measured as an important determinant of *Pseudomonas aeruginosa* pathogenicity, which have been implicated in virtually every human infection especially with nosocomial type and particularly recalcitrant to antibiotic compounds and can persist despite sustained host defenses. On another hand, the present study was the first report which investigates about the *pslÁ* gene in Iraq that has an important association with the ability biofilm formation regardless as strong or moderate in *P. aeruginosa* isolates recovered from variety infections. Also, the current study suggests the need to develop and to introduce a novel agents like-drug for biofilm mode of pathogens eradication as a part of clinical care, especially for patients suffered with diversity nosocomial infections.

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