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Biofilm Formation and Detection of *pslÁ* Gene in Multidrug Resistant *Pseudomonas aeruginosa* Isolated from Thi-Qar, Iraq

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Abstract: *Pseudomonas aeruginosa* is considered as one of the major causes of hospital-acquired infections due to its high antibacterial resistance. Biofilm formation is a well-known pathogenic mechanism in *P. aeruginosa* infections. The detection of polysaccharide *pslÁ* gene can be important for biofilm formation by this bacteria. Therefore; A total of 96 swab sample were collected from burn and otitis infections at the two hospitals in THi_Qar province, Iraq, there were just 93 samples gave positive growth. By differential and selective media(McChonky agar,Cetrimide agar at 44°C, King A agar), biochemical tests(gram staining, oxidase, triple sugar iron agar and others) and growth at 42°C to detection and diagnosis the isolates as *Pseudomonas* species, followed by APi 20E assay supported by genotypic determined of *16S rRNA* which proved there were 37(39.7%) identical isolates as *P. aeruginosa*. Antibiograms susceptibility test was performed via agar disk diffusion method were isolates subjected to 12 different items of antibiotics appeared that 12 and 4 isolates were multidrug resistant of burns and otitis respectively, with 3 isolates were pan-drugs resistance from burns. The ability of producing biofilm was examined by two phenotypic methods were Congo Red Agar and Crystal Violet Microtiter Plate Assay, the results showed that 32/37 (86.49%) isolates were slim adhesion formed in Congo red test, also there were 31/37 (83.78%) isolates produced biofilm formation, there were 43.47% and 21.42% strong biofilm formation from burns and otitis respectively by second method, all strong biofilm formation isolates were multidrugs resistance isolates. The detection and prevalence of the *pslÁ* genes among the isolates was determined by conventional monoplex polymerase chain reaction (PCR), the outcome exhibited that 34/37(91.89%) isolates carriage the *pslÁ* gene, also one and two from burns and otitis isolates respectively didn't carry this gene. The recent report is the first study with biofilm *pslÁ* gene in Iraq, and concluded that *pslÁ* gene harbored in most *P.aeruginosa* isolates even strong, moderate and weak biofilm but in different grade that mean there are another genes can contribute in biofilm synthesis must be investigated.

Key word: *Pseudomonas aeruginosa*, Antibiogram, Multidrug-resistance, Biofilm, *pslÁ* gene .

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Introduction:

The formation of biofilms facilitates chronic bacterial infections and reduces the efficacy of antimicrobial therapy(1). The Gram-negative pathogen *Pseudomonas aeruginosa* is a model organism for

biofilm studies and causes both acute and chronic infections by exploiting deficiencies in host immunity. *P. aeruginosa* is thought to exist as a biofilm during infections of the cystic fibrosis (CF) airway(2), in acute burn wounds, and in chronic suppurative otitis media(3). Biofilm formation in *P. aeruginosa* is regulated by a complex network of signals that includes small molecules, two-component systems, small RNAs, and nutritional cues(4).This species is leading to cause the nosocomial infections and is responsible for 10% of all hospital-acquired contagion(5). Infections caused by *P. aeruginosa* are often severe and life threatening and are difficult to treat because of the limited susceptibility to antimicrobial agents and the high frequency of an emergence of antibiotic resistance during therapy, thus resulting in severe adverse outcomes(6). Natural resistance of *P. aeruginosa* to several group of antibiotics, and the refractory to disinfectants together with the ability to biofilm formation make this bacterium responsible for high rates of morbidity and mortality(7). This pathogen produces three major exopolysaccharides (EPS) found within the matrix: alginate, Pel, and Psl(8). The charge-neutral exopolysaccharide Psl is playing a role in both cell to cell, and cell to substrate attachment(9)and(10). Billings *et al*(2013)(11)showed that polysaccharide, Psl, provides an added defense for *P. aeruginosa* biofilms against antimicrobials of different properties for young biofilms, and *P. aeruginosa*, *E. coli*, and *S. aureus* species that lack Psl take advantage of the protection offered by cells producing Psl. The *psl* gene cluster as, operon; comprising 15 co-transcribed genes *pslÁ* (PA2231) to *pslO*(PA2245) encoding proteins involved in EPS biosynthesis, as playing an important role in biofilm formation by non-mucoid *P. aeruginosa*(12)and(13), the first gene *pslÁ* is the most importance role in biofilm formation and on the regulation of the entire psl operon in planktonic and biofilm cells(14). Therefore; the goal of the recent work dedicated to investigating prevalence *pslÁ* gene in pre-detected multidrug-resistant with biofilm formation among clinical pathogenic samples of *pseudomonas aeruginosa*. isolated from some patients with severe burned and chronic suppurative otitis media(CSOM).

Materials and methods

Sampling:

Totaly of 96 swab samples distributed between; burn(n=53) and ear infections(n=43) swabs; from both "independent burns unit in Al-Hussein General Teaching Hospital", and; "Ear, Nose and Throat Consultant of Al-Habbobi Teaching Hospital for General Surgery and Fractures"/Thi_Qar province (about 365km faraway south of capital Baghdad), Iraq, respectively, compiled during mid-April/2016 to the terminus of September2016,patients included both male and female,different ages, diverse local regions and together urban and rustic habitat. And according to Murray and Baron(2007)(15),the transportation of all specimens were immediately transferred via disposable sterile cottonswabs(duplicate for each sample) 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)(16) starting with cultivating on rich, differentiation and selective media, included; 5% Blood sheep agar, Nutrient agar(N.A), MacConkey agar, Cetrimide agar, King A agar , in conjunction with traditional biochemical tests comprised; gram staining, oxidase, catalase, triple sugar iron agar(TSI), oxidation/fermentation glucose, growth and non-growth at 42°C and 4°C respectively were performed to characterize *P. aeruginosa* colony morphologically, cell microscopically and metabolically. ByAnalytical Profile Index (API) 20E Kit(BioMerieux -France)microtubules enzymatically tests to support strains identification, finally the molecular detection using *16s ribosomal RNA* boosted by conventional PCR technique standard on Persing *et al*.,(2016) (17), for obtaining pure isolates diagnostic at a species level, which stored in Brain Heart Infusion(BHI) both; slant with agar and broth contain 30%glecerol at refrigerator tmperatuere. and -20°C respectively, for next experiments.

Antibiogram test:

The disk diffusion susceptibility method for antibiogram testing(Kirby-Bauer method) was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines(18). Antibiotic discs (*Bioanalyse* – Turkey) used in this study included Ciprofloxacin (CIP), Levofloxacin (LEV), Amikacin (AK), Gentamicin (GN), Netilmicin (NET), Ceftazidime (CAZ), Cefepime (FEP), imipenem (IPM), Piperacillin(PRL), Aztreonam (AZT), Ticarcillin (TIC), and Ticarcillin/Clavulanic acid(TCC). So two or three identical pure colonies of *P. aeruginosa* was sub-cultured overnight on nutrient agar (N.A) plates at 37°C, re-suspended with 3ml deionized distilled water(d.d.w), the bacterial suspension was used as the inoculum adjusted the turbidity at a McFarland standard no. 0.5 (1.5 x 10^8) colony-forming units (cfu)/ml, next inoculate the dried surface of an Muller Hinton Agar(MHA) plate by streaking the swab over the entire sterile agar surface, repeat this procedure by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. then; 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(18). Multidrug resistance (MDR) was defined as resistance to at least one item from every different three antibiotic categories, as described by the international expert proposal for interim standard definitions for acquired resistance(19).

Congo red agar (CRA) assay as primary phenotypic detection of isolates ability to form adhesion slime

The initial determination for capability of *P. aeruginosa* isolates to produce adhesion slim was determined and the results interpreted by the CRA method described elsewhere(20), for this assay to prepare 250ml of this media; 9.5gm, BHI broth,12.5gm sucrose,2.5gm agar and 0.2gm Congo

red powder. Following autoclaved, the concentrated solution was added to the autoclaved brain heart infusion agar with sucrose, which was previously cooled to 55°C.Finaly, isolates test were cultivated in streaks on CRA plates, and incubate at 37°C for 24–48 hour aerobically. CRA-positive(Biofilm formation) strains appeared as black colonies, while CRA-negative(Non-biofilm) strains remained red.

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 worked of(21). In brief; adequate pure , three to four well-isolated identical colonies, grown overnight on tryptic soy agar(TSA) were suspended in a tryptic soy broth (TSB) supplemented with4% glucose, in new sterilized 5ml test tube diluted 1:100. After that, aliquots of (0.2 mL per-well) of each isolate suspension were then inoculated into eight- wells and wells containing un-inoculated(without bacteria) TSB media served as a negative control, followed by incubated overnight at 37°C. Next, the contents of the wells discarded, that each well is washed three times with 200 µL of phosphate-buffered saline (pH 7.2). Subsequent, the plates should be drain. Then, fixation with 160 µL of 99%methanol for 20 min, next the microtiter plates left to air dry overnight in an inverted position. Sequent, The adherent biofilm layer formed in each well was stained with 165µL of 1% crystal violet tincture, for ¼ hour at room temperature(RT). Later, washing and drying microtiter plate, the dye bound to the adherent cells was dissolved by added 180 µL 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 production was done according to the criteria of Stepanovic *et al*.,(2000) (22) as in table(1).

Table (1) Classification strength scope of biofilm formation.

OD values	Biofilm ranking
OD < ODc*	Non
ODc < OD < 2xODc	Weakly
2xODc < OD < 4xODc	Moderate
4xODc < OD	Strong

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

Total *P. aeruginosa* DNA extraction:

An aliquot of 1.5ml overnight reactivated growth in LB broth was added in 1.5ml 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, Qualitatively 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(2001)(23). Finally, the genomic DNA was conserved at -20°C in chest freezer 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; 12.5 µL of premixed ready master mix which mixed with forward and reverse primers(10 pmol/1µl) each, the specific primers of genes provide from *Alpha DNA*(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 as in table(3). Amplifications were carried out in Gradient- Veriti ™ 96-Well Thermal Cycler(AB_ Applied Biosystems- Singapore) thermo-controller.

Table (2): List primers for PCR detection of the biofilm *pslA*& *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	*Psedo. aeruginosa detection*	956	189-206 1124-1144
pslÁ -F1 *pslÁ* -R1	CACTGGACGTCTACTCCGACGATAT GTTTCTTGATCTTGTGCAGGGTGTC	55	Psl Expolysacchride	1119	1-25 1095-1119

***Position and size relative to *16S rDNA* and *pslA* sequence of *P. aeruginosa* PAO1 used SnapGene 1.5.3 program.**

Table (3): Components solution of PCR reaction for detecting *16S rRNA* and *pslÁ* genes.

Ingredients	Volume (µl)
Master mix (2X Taq Mix- Dongsheng Biotech,China)	12.5
Forward primer	1
Reverse primer	1
Template genomic DNA	1
nuclease-free double distilled water (ddH2O)	9.5
Total Volume	25

A thermal step program was used, including the following parameters: firstly; for *16S rRNA*(1535 bp) amplification was used to diagnostic *Pseudomonas*, as *aeruginosa* species cited from(24) , the reaction program was illustrated in table(4), 2 min at 95°C for an initial denaturization ; 25 cycles of 20 s of denaturation at 94°C, 20 s with the appropriate annealing temperature at 58°C and 40 s of extension at 72°C. A final extension of 1 min at 72°C was applied.

Table (4): 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	

Secondly; with the biofilm production *pslÁ* (1437 bp) gene, the thermal protocol amplified based upon(25) demonstrated in table(5). was an initial incubation at 94°C for 10 minutes, followed by 30 cycles of one minute denaturation at 94°C, 30 s an optimization annealing at 55°C and one minute extension at 72°C followed by 10 minutes at 72°C final extension.

Table (5): 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	

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(23).

Statistical Analysis:

All experiments were performed at least in duplicate or triplicate, also averages and percentage of values are recorded in the tables and figures.

Results and Discussion:

Sampling, isolation and identification of *P.aeruginosa* by traditional methods

The common microbiological detection methods appeared that of the 96 clinical specimens, there were just three given no growth, while 93 manifested positive culture, of this total there were 39 indicated as *Pseudomonas aeruginosa* , the results of media growth and biochemical tests listed in table (6).

Table (6): 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, figure-(1), the results revalded that of 93 there were 37(39.7%) specimens burn, n=23/(62%) and otitis, n=14/(37.8%) restricted with *P. aeruginosa* species, Finally, the genotypically analysis via PCR reaction with specific 16S ribosomal RNA primer in figure(2), finishing the confused about diagnosis and confirm the API 20E test proved that 37 specimens were the accurate conclusive outcome and within *Pseudomonas aeruginosa*. The result of burn specimens in recent search somewhat agree in both diagnostic and number with Iraqi results of (26)that there isolation showed of 111 there were 25 *P. aeruginos*.

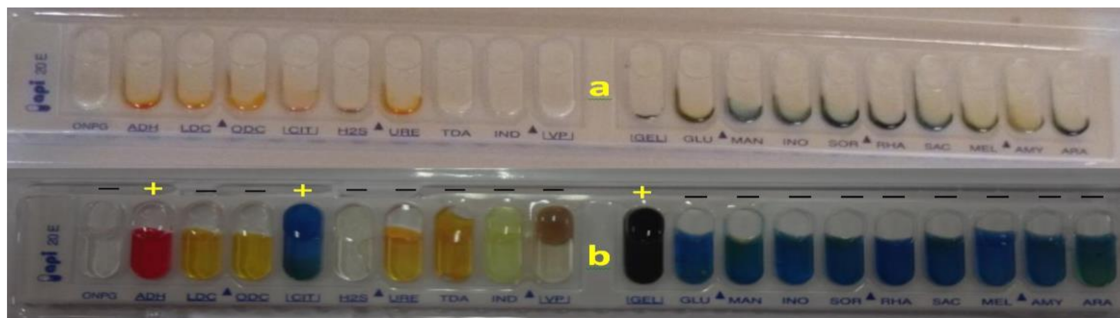


Figure (1): Isolates diagnosis via API 20E microtubules strip test, a-Before use& b-After use.

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 (27).

Molecular detection by 16S rRNA gene. Just 2 isolates didn't match with 16S rRNA primer, that 37 isolates were belong *P. aeruginosa* accurately,

showed in figure-(2), genotypic detection is a precisely& favorable for bacterial diagnosis at species level other than traditional technique especially with *P. aeruginosa* confirmed from(24).

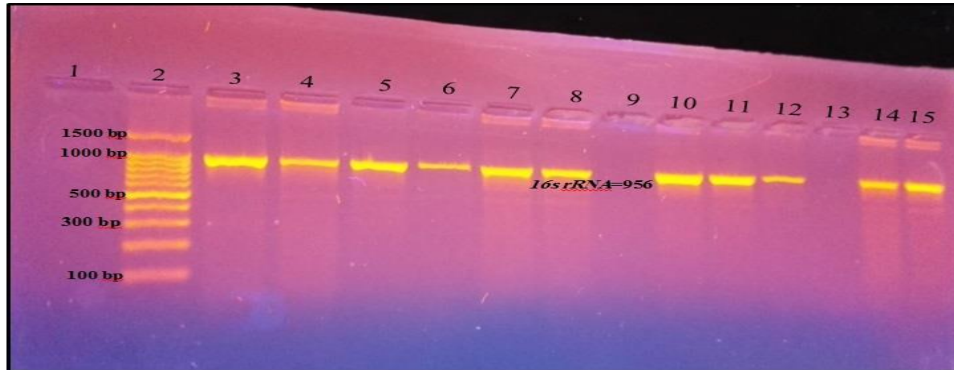


Figure (2): Gel electrophoresis of amplified PCR product of *16S rRNA* gene(956bp) in monoplex PCR at 70v for 90 min in 1.7 % agarose , TBE (1x) , stained with ethidium bromide . Lane(2): DNA ladder (100bp),lane(1) negative control(without DNA) , all the lanes were positive for the target gene except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

Antibiogram assay:

Of 12 antibiotic items *P. aeruginosa* exhibited diversity in resistance phenomena, the burn isolates were a high rate of resistance against Netilmicin, Aztreonam, Ticarcillin, and Ticarcillin/ Clavulanic acid , while continued with burn specimens the rate impedance were something became less progressively with rest, antibiotics, down to Ciprofloxacin, Imipenem and Amikacin, they were most effective, respectively, and no more than 12/23(52%) were Multi-DR of these 3 were resistant to all the tested agents(pan-drug resistance,PDR), figure-(3). While, the isolates of otitis were just 4/14(28.6%) isolates MDR, the other appeared diversity in antibiogram reluctance, so the histogram in figure-(4) summarizing the total percentage (%) antibiogram resistance outcome of *P. aeruginosa* isolates against each antibiotic.

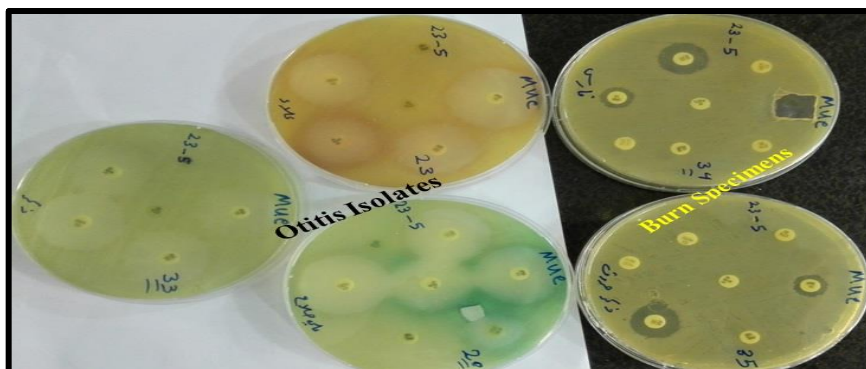


Figure (3):Antibiogram test of burn and Otitis patterns.

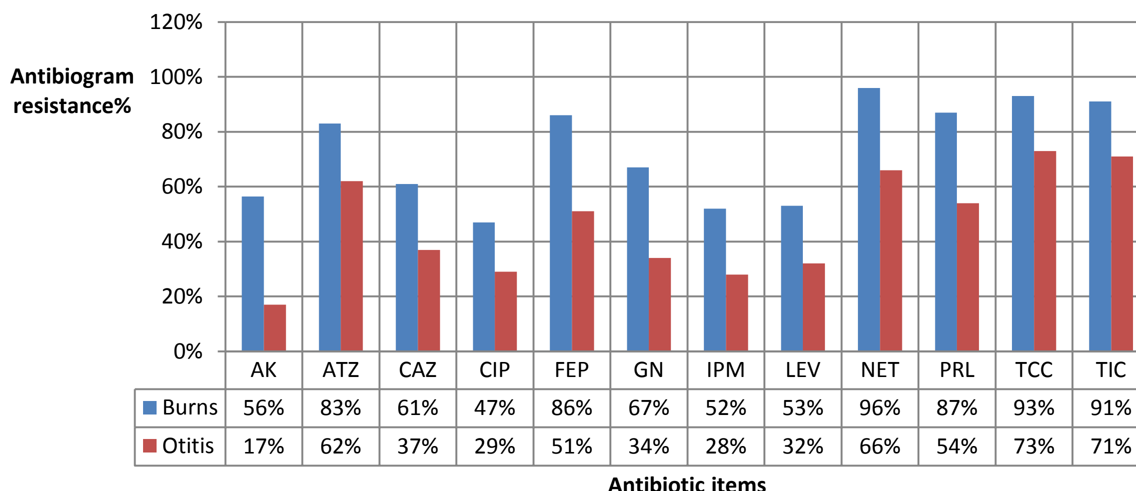


Figure (4): Histogram of the percentage of multi-DR isolates against 12 antibiogram items .

From the results elucidated in histogram above not surprising that the burn isolates in general more resistant than otitis isolates, this attributed to the patients with burn injuries are frequently exposed to antimicrobial agents throughout their hospitalization more than otitis illness, increasing the likelihood of colonization or infection with drug-resistant organisms (28). Also the results of the present research compared with some pervious investigation recorded a variable percentage of *P. aeruginosa* antibiogram resistant as with studying of Corehtash *et al*., (2015) (29) was a high rate of resistance against Ciprofloxacin (93.7%), Ceftazidime (82.6%), Amikacin (82%), Imipenem (79.2%), and semi- similar with Aztreonam (86.8%), Piperacillin (85.4%), but the least resistance was seen with Gentamicin (11.1%). Over all, other previous studies agreed on the increasing of *P. aeruginosa* resistance due to produce a chromosomally encoded AmpC beta lactamase, which can hydrolyze antipseudomonal penicillins, monobactam, and third-generation cephalosporins. Also the efflux pumps are an important mechanism of multidrug resistance to quinolones by mutations to the chromosomally mediated topoisomerases II and IV, antipseudomonal penicillins, cephalosporins 4^{Th} generation, and sometimes aminoglycosides may be mediated by outer membrane impermeability or by aminoglycoside modifying enzymes (30). So the differences and diversity in characteristics and properties of antibiotic resistance among bacterial infections explained by Geisinger and Isberg suggested hospital-borne diseases are the outcome of a complex interplay of several dynamic factors operating at the level of the infecting microorganism, the host patient, and the hospital environment. These include the pathogenicity, drug resistance, and environmental persistence of the microbe; the immune status and microflora composition of the human host; and nosocomial interventions such as antimicrobial therapy. As these factors interact, the rapid and

widespread rise in intractable of bacterial multidrug-resistant (MDR) with responsible for nosocomial diseases constantly evolve(31).

Detection adhesion capability of isolates by screening of Congo red agar

The primary phenotypic estimated of biofilm formation accomplished by Congo red agar test figure-(5), so the total black or light black color colonies were (32/37=86.49%) biofilm formation(spanned between strong to weak), distributed between (21/23=91.3%) and (11/14=78.57%) of burn and otitis isolates respectively. The rest of isolates were red or pink colour indicated to non-biofilm formed.

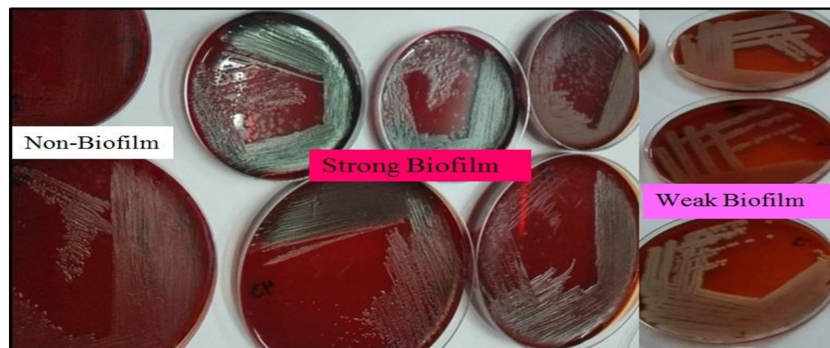


Fig.(5): Congo Red Agar assay evaluated phenotypically qualification of slim adhesion among *P. aeruginosa* isolates.

The result of this work agreed with outcome investigation of Rewatkar and adher, (2013) (32) when of 30 nosocomial infection *P.aeruginosa* isolates there were 27(90%) and 3 (10%) biofilm and non-biofilm formation respectively. The CRA test is a primary satisfactory biofilm determination but not accurate enough, therefore; the needing for supported other method must be adopted (33).

Quantitative measurement of biofilm assemblage.

Quantitative biofilm determination using the micro-titer plate assay figure-(6), revealed that 31/37 (83.78%) isolates produced biofilm and the remaining 6 isolates were non-biofilm producers that 2 and 4 from burn and otitis respectively. The burn isolates were more with strong biofilm with adequate to be significantly than otitis strains, the large number of total isolates were strong and moderate biofilm grade, and the weakly biofilm were more with otitis, the figure- (7) the histogram show the complete results. although this result less than in dominant percentage that founded by (29) was 92.4% of *P. aeruginosa* isolates that form the biofilm detected by the same test, but remained in higher % of biofilm formation than non-formation isolates. 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 (1).

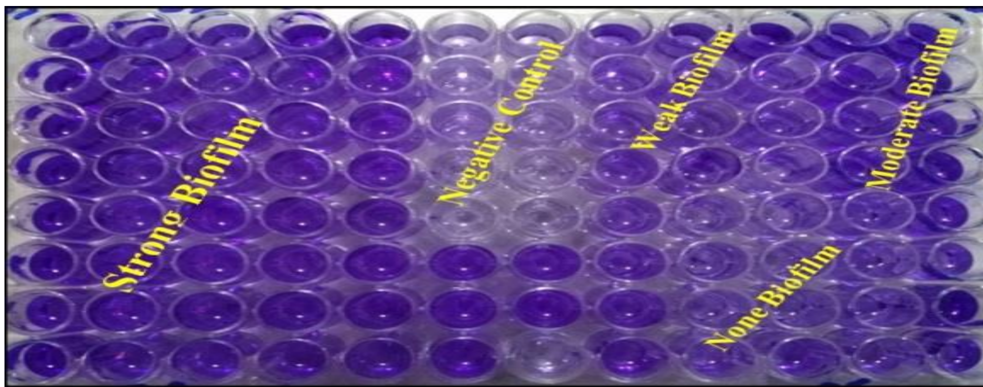
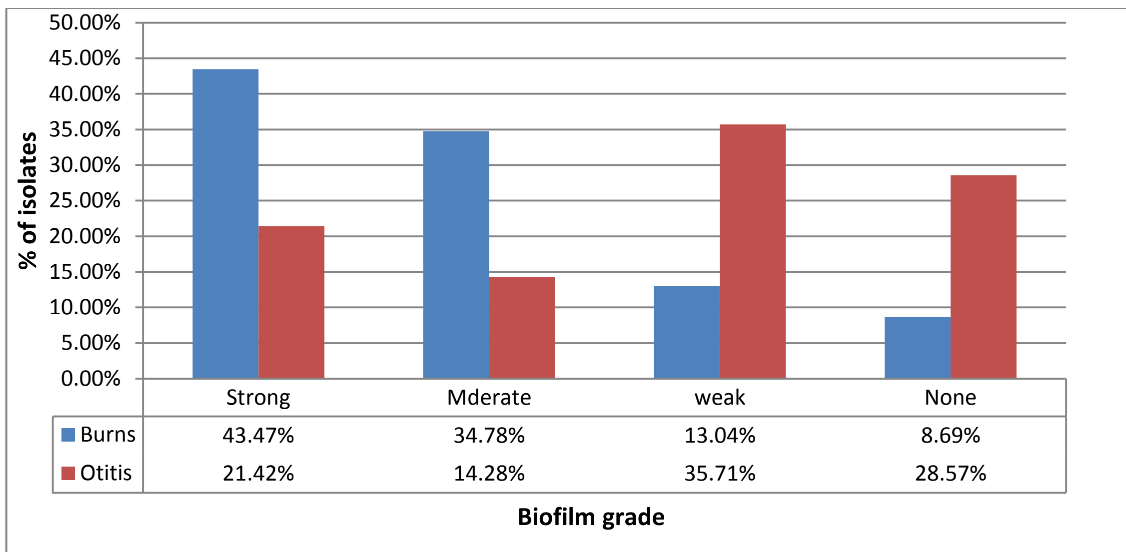


Figure (6): Biofilm quantitative estimated by Micro-titer tissue culture 96-well flat bottom



	Strong	Mderate	weak	None
Burns	43.47%	34.78%	13.04%	8.69%
Otitis	21.42%	14.28%	35.71%	28.57%

Figure (7): Histogram of biofilm formation for *P. aeruginosa* isolates by micro-titer plate assay .

P. aeruginosa DNA extraction:

Determination of DNA concentration and purity were done using the Nano drop device previously mentioned. The results indicated that DNA concentrations of the extracts were variable ranging from 153.6 ng/ µl to 422.8ng/ µl that mean the average was 288.2 ng/ µl, figure-(8) obvious bands of gel electrophoresis for total genomic DNA of isolates. It was also observed that the purity of DNA extracts washigh ranging from 1.73 to 1.87. The output dependently on culturing methods, bacterial category, amount of pellet and type of extraction kit, all these have an affected on quality and properties of nucleic acid. That, most of molecular practical methods indicated to more easy and adequate of DNA extraction from gram negative than positive and this applies to recent ready extraction kits (34).

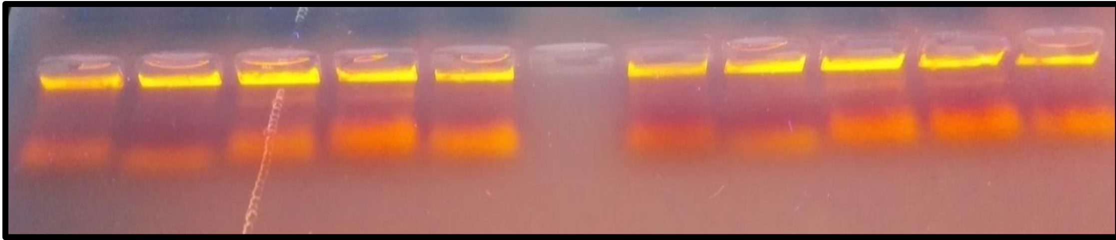


Figure (8): Gel electrophoresis(100V at ¼ h) for hall genomic DNA of *P. aeruginosa* isolates.

PCR technique and genotypic detection of biofilm *pslÁ* gene:

The amplification product of *pslÁ* gene for a number of *P. aeruginosa* isolates (1119 bps) is shown in figure (9), there were 34/37(91.89%) carriage the *pslÁ* gene, one and two from burns and otitis isolates respectively didn't carry this gene.

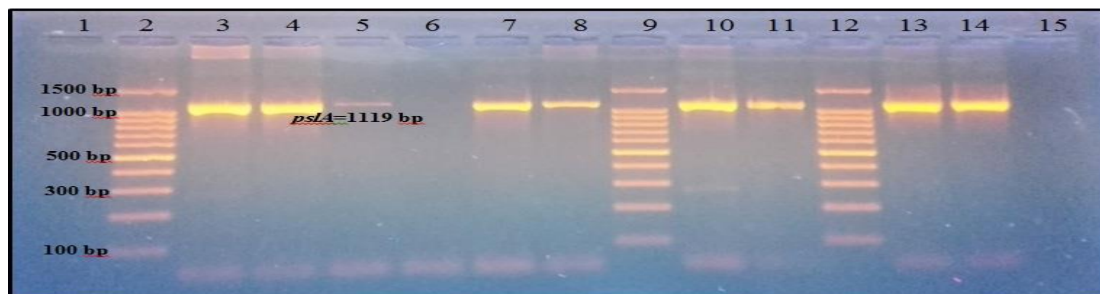


Figure (9): Gel electrophoresis of amplified PCR product of biofilm *pslA* gene(956bp) in monoplex PCR at 70v for 90 min in 1.7 % agarose , TBE (1x) , stained with ethidium bromide . Lane (2,9and12): DNA ladder (100bp), lane(1) negative control(without DNA) , all the lanes were positive for the target gene except the lanes from (6-15) were negative result.

The result of prevalence and harboring for *pslA* gene in *P. aeruginosa* isolates of recent work was high percentage than in Iranian studies of (35) and (36) were 42.9% and 52% respectively. Also in this research there was a relationship observed between antibiotic resistance profiles with both biofilm-positive and biofilm-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. According to Gaddy and Actis, the multidrug resistance correlates with the ability to form biofilm on abiotic and biological surfaces (37), also the work of Drenkard and Ausubel (2002), were found that the antibiotic-resistant variants of *P. aeruginosa* had high ability to form biofilm both in vivo and in vitro(38), which by Bolaji *et al*., (2011) explicated that, the transferring of such high-resistance isolates can occur in environments such as groundwater or somehow in healthcare centers and clinical instruments where it becomes a potential risk for the human health (39). To evaluate the essential

role of *pslA* in biofilm formation, study of Overhage *et al*., generated a nonpolar isogenic *pslA* knockout mutant of *P. aeruginosa*. The observation of this *pslA* knockout 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 (14). In another work by Ghafoor *et al*.,(2011) they 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 (8). From suggestion of (35) emphasized that it seems the *pslA* gene had association with biofilm formation, since it was widely distributed among the biofilm-producing isolates. These results showed that the *pslA* gene was an important factor to form biofilm. However, since this gene was found in all the biofilm producers including strong and weaker within burn and otitis isolates, perhaps there were other genes or factors that played role in forming biofilm. Therefore, we should consider other genetic and phenotypic factors as well, which afford for future studies.

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 are 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 burn and otitis infection. Also, the current study suggest 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 from burns and SCOM.

Ethical considerations:

The study got approved by The Graduate Studies and The Academic Research Committees at The Faculty of Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, Iraq. In addition, The agreement of patients or their parents was taken when specimens compiled and supervised by medical staff in the hospitals mentioned in this study. Acknowledgement

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