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Original article

Correlation between biofilm formation and multidrug resistance in clinical isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Background: *Pseudomonas aeruginosa* (*P. aeruginosa)* is an opportunistic multidrug resistant (MDR) organism with high morbidity and mortality rate among hospitalized and immunocompromised patients. This is attributed to its natural resistance in addition to biofilm forming capacity. We aimed to detect the prevalence of biofilm between *P. aeruginosa* isolates and its relation to MDR. **Methods**: 191 *P. aeruginosa* isolates were collected from different clinical specimens after taking a written consent from the patients. The isolates were identified by standard microbiological methods. The disc diffusion was used to test the sensitivity of isolates to various antibiotics. Detection of biofilm phenotypically and genotypically was done. **Results:** Antibiotic susceptibility pattern of *P. aeruginosa* clinical isolates to different antibiotics showed that (56.5%), (52.9%), (47.6%), (47.1%), (47.1%), (46.6%), (44.0%), (43.5%), (40.8%) of *P. aeruginosa* isolates were resistant to CN, AK, AMC, ETP, IPM, PTZ, CAZ, CIP and CTX respectively. 69.1% of isolates were MDR. 84.3% were biofilm -producers by phenotypic method. The percent of genes encoding biofilm among 191 *P. aeruginosa* strains were 62.3%. 46.6% of the isolates presented all three genes "*algD +, pslD +, pelF +"*, 12.6% were *"algD+, pslD +"* genes, 3.1% were *"pslD+"* gene while 37.7% did not present any gene "*algD−, pslD−, pelF–*. **Conclusion:** Biofilm forming *P. aeruginosa* showed high MDR level and biofilm production is associated with presence of *algD /pslD /pelF* genes.

Introduction

Pseudomonas aeruginosa is an opportunistic organism that cause different acute and chronic diseases in immuno-compromised individuals like patients suffering from cystic fibrosis, tumors, post-operative, severe burns or infected by human immunodeficiency virus (HIV) and those hospitalized in intensive care units [1]. Emerging of multi drugs resistant strains is a major problem leading to high morbidity and mortality. Beside high intrinsic resistance of *P. aeruginosa* to antibiotics, its biofilm forming ability renders treatment of *P. aeruginosa* infections very difficult [2].

Biofilm formation allows bacteria to survive inside the host's body and withstand harsh environments. It is considered one of the major virulence factors as it permits cumulative bacterial growth by adhering to surfaces by forming self-secreted matrix extracellular polymeric substance (EPS). Also, it protects bacteria from host immune system [3]. On the other hand, horizontal gene exchange is greatly enhanced in biofilms since resistant bacteria can transmit genes of resistance to

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other one. Moreover, numerous bacteria inside biofilm become metabolically inactive so, it will remain unaffected by antimicrobials as they act on metabolically active bacteria [4].

The EPS matrix limits diffusion of some antibiotics into the biofilm. Therefore, biofilm has an important role in developing antibiotics resistance [5]. The EPS is formed of polysaccharides, proteins, and nucleic acids allowing the organism to thrive in difficult conditions such as undesirable pH, humidity, and temperature [3]. Biofilm forming bacteria can survive in living tissue, prosthetic medical instruments, and on solid surfaces [6].

There are several factors that encourage biofilm formation and growth for example presence of minerals such as calcium, copper, and iron. [7]. There are at least 3 genes, alginate, pellicle *(Pel)* and polysaccharide synthesis locus (*Psl)* that involved in producing biofilm [8]. Alginate is mainly produced by strains isolated from the lungs of cystic fibrosis (CF) patients and its synthesis is mediated through the *alg ACD* operon.

Polysaccharide synthesis locus plays a role in interactions between cells and surface in forming biofilm, so it has an essential role in initiating and protecting biofilm structure [9]. This exopolysaccharide also confers resistance of *P. aeruginosa* biofilms to antibiotics and phagocytic cells [10]. The *psl* operon consisting of 12 *psl* genes *(psl A-L*) necessary for *Psl* production [11]. The *pel* operon enables *P. aeruginosa* to form a sheet of cells above the surface of a standing culture [12]. *Pel* is an exopolysaccharide consisted of several sugars [13]. It is controlled by *(pel ABCDEFG)* [14].

In order to limit the spread of multidrug resistant (MDR) strains, decrease severity of infections by *P. aeruginosa*, reduce mortality and hospitalization rates as well as the economic burden associated with such resistant pathogen, this study was carried out to detect the pattern of antimicrobial sensitivity, the biofilm phenotypic and genotypic properties and study the relation between biofilm production and resistance to various antibiotics in *P. aeruginosa* strains recovered from patients in Zagazig University hospital.

Patients and Methods

Study design and participants

This cross-sectional study was carried out in Zagazig university hospitals, Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University throughout 8 months from August 2020 to March 2021. This comprehensive study included all patients suffering from infections caused by *P. aeruginosa* such as burn, respiratory tract infections and urinary tract infections.

Ethical approval

The study was approved by Zagazig University Institution Review Board (ZU-IRB) (Approval code 6735). This study was done regarding The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was taken from the patients or their relatives.

Microbiological work

Clinical samples collection and *P. aeruginosa* identification: 191 *P. aeruginosa* isolates were recovered from aseptically collected clinical samples. Samples included endotracheal aspirates, urine, burn, wound, sputum, pus, csf and blood. History of patients included age, sex, and previous intake of antibiotics were stated. Standard microbiological and biochemical procedures, such as Gram stain, culture characteristics of colonies, growth on cetrimide and MacConkey agar, nutrient agar for pigment production, growth at 42 °C, several biochemical tests like (oxidase, catalase, citrate utilization, reaction on triple sugar agar and test for motility), were used in the laboratory to identify *P. aeruginosa* isolates [15].

Antimicrobial susceptibility testing: According to the Clinical and Laboratory Standards Institute (CLSI) standards, isolates' susceptibility to various antibiotics was tested using the disc diffusion agar method on cation adjusted Mueller–Hinton agar **(Oxoid, UK)**. Antibiotic disks **(Oxoid, UK)** used in the study were "ceftazidime (CAZ, 30µg), cefotaxime (CTX, 30ug), amoxicillin clavulanic (AMC, 20/10µg), piperacillin/tazobactam (PTZ, 100 µg/10µg), ciprofloxacin (CIP, 5µg), gentamicin (GM, 10µg), amikacin (AK, 30 µg), imipenem (IMI, 10µg), and ertapenem (ETP, 10ug)". *Pseudomonas aeruginosa*ATCC®27853 was used as a quality control strain **(American Type Culture Collection [ATCC], Manassas, VA, USA)** [16]. *Pseudomonas aeruginosa* found to be resistant to more than one antibiotic in 3 or more groups of antimicrobial was recognized as MDR *P. aeruginosa* (MDR-PA) [17].

Phenotypic detection of biofilm: Microtiter plate assay, as reported by **Stepanovi et al**. [18] was used to assess biofilm formation quantitatively with some changes. A *P. aeruginosa* overnight culture was

prepared to a 0.5 McFarland turbidity standard. Suspensions were diluted 1:100 in 200 mL tryptic soy broth (TSB) with one percent glucose (**Oxoid, UK**) before being transferred to (presterilized, flat bottomed, polystyrene,96-well microplates). After 24 hours of incubation at 37°C, the wells were gently rinsed three times with sterile phosphate buffered saline (PBS, pH 7.3). The adherent biofilms were fixed for 15 minutes in 99 percent methanol, then the solutions were withdrawn, and the plate was left to dry in air. Biofilms were stained for 5 min at room temperature by 200 µL of 0.1 percent crystal violet **(Sigma Chemical Co., USA)**, and then washed with water and left to dry. Adding 200 µL of ethanol (95%) for thirty min destained biofilm in each well. Using a microtiter plate reader, at 570 nm, the optical density (OD) was determined (**BioTek, USA**). All of the trials were carried out in triplicate and for 3 times. A cut-off value (ODc) was defined as 3 standard deviations (SD) higher than the negative control's mean OD i.e Odc = negative control's average OD + (3 SD of negative control). The isolates were divided into 4 groups according to the OD: non-biofilm producer (OD < ODc); weak producer (ODc < OD < 2 × ODc); moderate producer (2 × ODc < OD < 4 × ODc); strong producer (4 × ODc < OD).

Genotypic detection of biofilm: By polymerase chain reaction (PCR), all isolates were screened for "*algD, pslD, and pelf",* biofilm-encoding genes, using primers specified in **table (1)** [19]. Using the DNA Extraction Mini Kit (i-genomic BYF, Korea), DNA was extracted from bacterial colonies. In a total volume of 20 µl, a PCR reaction was carried out with master mix (10 µl), Taq polymerase enzyme (0.8 µl), DNA extract (4.4 µl), and 0.8 µl of each forward and reverse primer (20 pmole). The following thermal conditions were used to amplify the three genes: 5 minutes at 95 °C, then 30 amplification cycles for 30s at 94 °C, for 40s at 60 °C, for 40s at 72 °C and lastly, at 72 °C for 5 minutes for elongation step. The products of PCR were detected by UV light after electrophoresis for 45 min on a 1.5 % agarose gel at 100 V.

Table 1. Primers used for the amplification of the genes coding for biofilm exopolysaccharides among *P. aeruginosa* isolates.

Gene	primer sequence (5''→3')	Size of amplicon (bp)
algD	F-CTACATCGAGACCGTCTGCC	593
	R-GCATCAACGAACCGAGCATC	
pelf	F-GAGGTCAGCTACATCCGTCG	789
	R-TCATGCAATCTCCGTGGCTT	
pslD	F- TGTACACCGTGCTCAACGAC	
	R- CTTCCGGCCCGATCTTCATC	369

Statistical analysis

All data were analyzed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation (SD) and range. For qualitative data, the Chi-Square test was employed to compare two groups. Kappa agreement was employed to measure the similarity between phenotypic and genotypic biofilm forming ability. All tests were two sided. $p < 0.001$ was considered highly statistically significant (HS) and $p \geq 0.05$ was considered non statistically significant (NS).

Results

A total of 191 *P. aeruginosa* isolates were collected from patients, their average age was 54.8±7.5 ranged from (40 to 70) years, of which 108 (56.5%) were males and 83(43.5%) females. Regarding distribution of *P. aeruginosa* in collected samples, most isolates (25.1%) were isolated from urine as shown in **figure (1)**.

Antibiogram of *P. aeruginosa* isolates to different antibiotics showed that 107 (56.5%), 101 (52.9%), 91 (47.6%), 90 (47.1%), 90 (47.1%), 89 (46.6%), 84 (44.0%), 83 (43.5%), 78 (40.8%) of P. *aeruginosa* isolates were resistant to CN, AK, AMC, ETP, IPM, PTZ, CAZ, CIP and CTX respectively as shown in **figure (2)**. Prevalence of MDR were 132(69.1%) isolates.

Phenotypic grading was estimated by the ability of *P. aeruginosa* isolates to form biofilm, 161 (84.3%) of the isolates were biofilm producers where 24 isolates (12.6%) were strong producers, 79

isolates (41.4%) were moderate producers, 58 isolates (30.4%) were weak producers and 30 isolates (15.7%) were non producer. Concerning the relation between biofilm formation and MDR, there were (120 out of 161)74.5% MDR isolates showing biofilm formation; 120 isolates showed MDR pattern were distributed as (23,73 and 24 isolates) produced (weak, moderate and strong) biofilm, respectively as shown in **table (2)**.

The frequency of genes encoding biofilm among 191 *P. aeruginosa* strains were 119 (62.3%). 46.6% (n=89) of the isolates presented all three genes "algD +, pslD +, pelF +", 12.6% (n= 24) were "algD+, pslD +" genes, 3.1% (n=6) were "pslD+" gene while 37.7% (n=72) did not present any gene "algD −, pslD −, pelf". Furthermore, phenotypic and genotypic properties of biofilm were used to categorize isolates into four groups.: biofilm + / gene + (n=119, 62.3%); biofilm -/ gene +(n=0, 0.0%); biofilm +/gene -(n=42, 21.9%) and biofilm -/gene - (n=30, 15.7%) **(Table 3)**.

There was statistically significant good agreement between detection of *P. aeruginosa* biofilm phenotypically and genotypically (kappa=0.47, *p*_value 0.001**) as shown in **tables (3,4)**.

Figure 1. Percentage of the *P. aeruginosa* isolates from the different specimen.

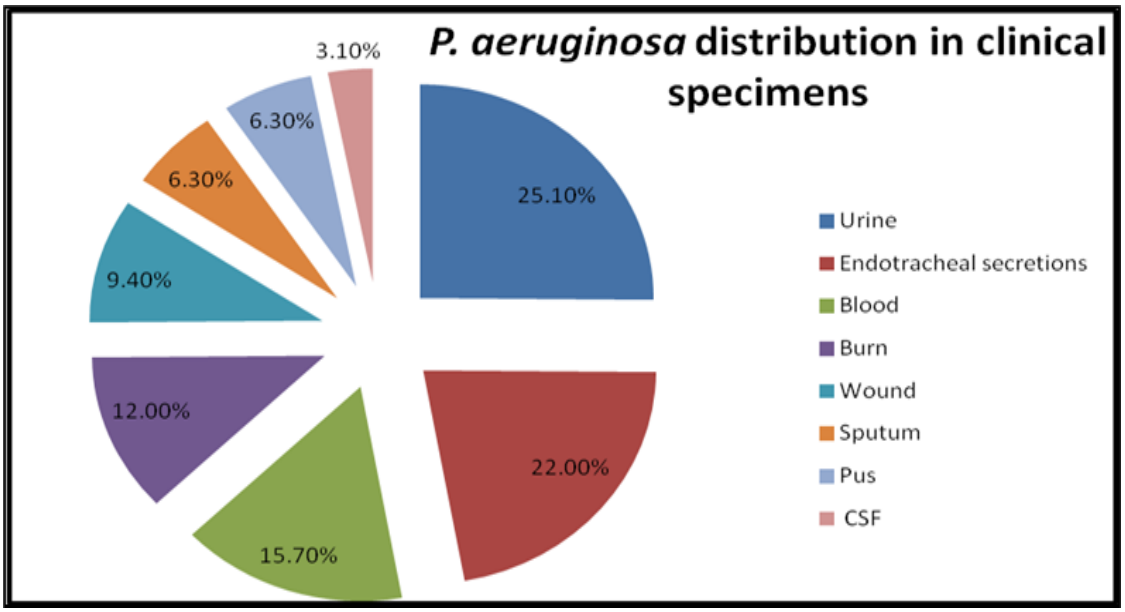

Figure 2. Antibiotic susceptibility of *P. aeruginosa* clinical isolates to different antibiotics.

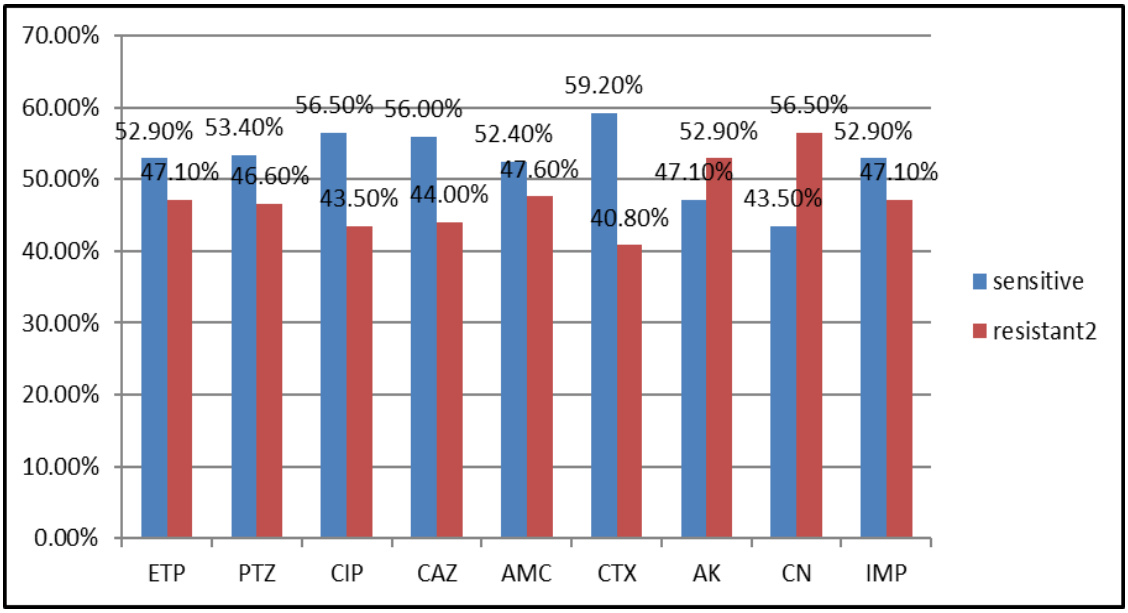

Figure 3. PCR amplification of biofilm-encoding genes in *P. aeruginosa* clinical isolates. Lane (M): DNA ladder 100 bp, lane (1): negative control, lane (2) and (4): positive for *pslD, algD, pelF* genes, lane (5): positive for *pslD, algD* genes, lane (3) and (6): negative cases.

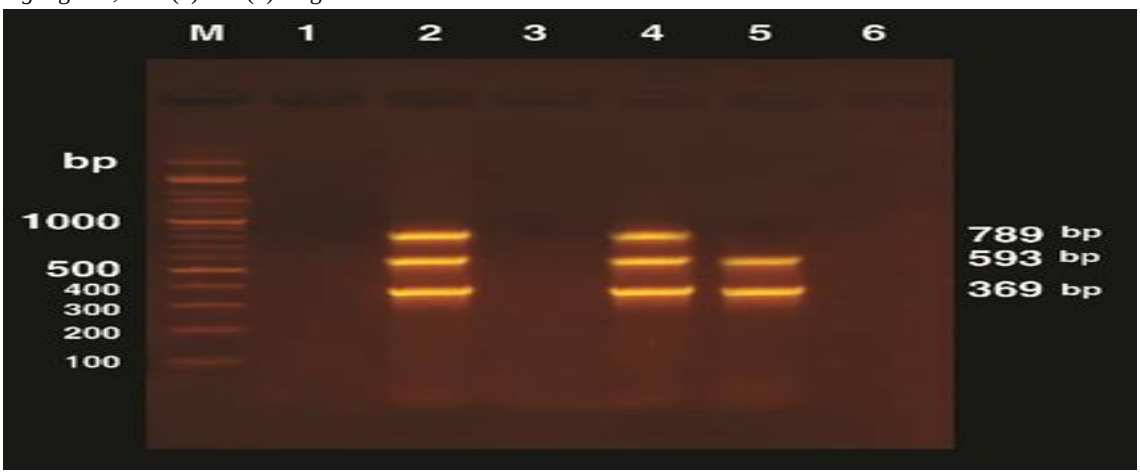

Table 2. Relation between biofilm forming and MDR among *P. aeruginosa* isolates.

Phenotypic pattern	MDR		*p*-value#
	No NO.=59 (30.9%)	**Yes NO.=132 (69.1%)**	
Non producer (n=30)	18 (60.0%)	12 (40.0%)	**0.001****
Weak (n=58)	35 (60.3%)	23 (39.7%)	**0.001****
Moderate (n=79)	6 (7.6%)	73 (92.4%)	**0.001****
Strong (n=24)	0.0 (0.0%)	24 (100.0%)	**0.001****

#=Chi square test, MDR=multiple drug resistance, **statistically highly significant difference.

Table 3. Relation between phenotypic and genotypic characteristics of biofilm among *P. aeruginosa* isolates.

Variables		Genotyping		Kappa	*p*-value
		Negative No= 72 (37.7 %)	**Positive No=119 (62.3 %)**		
Phenotypic	Non_producer (n=30, 15.7%)	30 (100.0%)	0.0 (0.0%)	0.47	**0.001****
	Positive (n=161, 84.3%)	42 (26.1%)	119 (73.9%)		

**Statistically highly significant difference.

Table 4. Agreement between phenotypic grading of biofilm forming and genotypic detection of *P. aeruginosa*.

Phenotypic pattern	Genotypic pattern		*p*-value#
	Negative No=72 (37.7%)	**Positive No=119 (62.3%)**	
Non_producer (n=30)	30 (100.0%)	0.0 (0.0%)	**0.001****
Weak (n=58)	12 (20.7%)	46 (79.3%)	**0.001****
Moderate (n=79)	30 (38.0%)	49 (62.0%)	0.9
Strong (n=24)	0.0 (0.0%)	24 (100.0%)	**0.001****

#=Chi square test ,**Statistically highly significant difference.

Discussion

Pseudomonas aeruginosa is an opportunistic human pathogen that causes life-threatening acute and persistent infections in people with weakened immune systems. Its ability to produce antibiotic-resistant biofilms is responsible for its highly endurance in clinical settings [2]. In the present study, the results of antimicrobial susceptibility pattern showed highest resistance of *P. aeruginosa* isolates towards; Gentamicin (56.5%), Amikacin (52.9%), Amoxicillin clavulanic (47.6%), Ertapenim and Impenim (47.1%). This is in agreement with **Banar et al.** [20] who reported that more than 90% of the isolates were resistant to amikacin, gentamicin, cefepime and meropenem and were MDR. Also, **Mohamed et al.** [21] showed MDR in 53% of *P. aeruginosa* isolates with high resistance to gentamycin (62%), amikacin (56%) and meropenem (60%). On the other hand, **Abdulhaq et al.** [22] showed that *P. aeruginosa* isolates had the maximum resistance to Ceftriaxone (94.23 %), Meropenem (92.30 %), Imipenem (90.38 %) and Aztreonam (84.61 %).

The prevalence of isolates with MDR pattern in our study was (69.1%) which was closer to the results obtained by **Helmy and Kashef** [23], **Yekani et al** [24] **and Talaat et al** [25]. (65.4%., 65% and 59.8%) respectively. On the other hand, Different percent was reported by other studies (76%,87%, and 38.46 %) by **Mahmoud et al.** [26] **Banar et al.** [20] and **Abdulhaq et al.** [22] respectively.

Probably, different results of antimicrobial resistance levels among multiple studies are most likely due to variances in antibiotic usage patterns in different locales. Furthermore, the high occurrence of MDR *P. aeruginosa* strains can be linked to the abuse of a wide spectrum of antimicrobials to manage nosocomial infections, or a mutation in *P. aeruginosa* genome. So, regarding the site of bacterial isolation, a suitable therapeutic regimen for treating *P. aeruginosa* infections should be chosen.

Concerning phenotypic detection of biofilm formation in our study, 161 (84.3%) of the isolates were biofilm producers where 24 isolates (12.6%) were strong producers, 79 isolates (41.4%) were moderate producers, 58 isolates (30.4%) were weak producers and 30 isolates (15.7%) were non producer. Similar results were reported by **Kamali et al.** [27] where 83.75% of isolates formed biofilm which was classified into :16.25% strong biofilm; 33.75% moderate biofilm; 33.75% weak biofilm producers, while 16.25% of isolates didn't form biofilm. Also, **Mahmoud et al.** [26] has reported (70.4%) of isolates were biofilm producers, 14.8% strong biofilm, 46.3%moderate biofilm and 9.3% weak biofilm producers, whereas, (29.6%) of isolates were non producers. These results are also in agreement with Banar et al. [20]. While **Elhabibi and Ramzy** [28] reported during their study in Egyptian hospitals that all isolates were biofilm producers; (90%) strong producers; (10%) moderate producers and (8%) weak producers. In this study, there was a significant correlation between MDR and biofilm production, where 74.5% of isolates producing biofilm showed MDR. These results were in agreement with other reported studies [24,26] which found that the bacteria in biofilm form are more resistant to antibiotics than in planktonic one

Our study detected a great percentage of genes that involved in biofilm production, being presented in 119 (62.3%) of *P. aeruginosa* isolates, where (*algD +/pslD +/pelF +*) was the most frequent pattern (46.6%). This is in consistent with **Kamali et al.** [27] who found high frequency of "*algD, pslD, and pelf"* genes (87.5%) in *P. aeruginosa isolates*. Similar results were reported by **Banar et al.** [20]. Different genes involved in forming the biofilm, were discovered by **Ghadaksaz et al.** [29] with 83.7% for *"pslA"* and 45.2% for *"pelA"*, and **Pournajaf et al.** [30] reported 89.5% for *"pslA"* and 57.3% for *"pelA"*, between *P. aeruginosa* isolates. However, no much information regarding the distribution of the "*pslD and pelf"* genes in various region of the world is available.*P. aeruginosa* isolates had statistically significant good agreement between phenotypic and genotypic detection of biofilm (kappa=0.47, *p* value 0.001**). There were 73.9 % out of 161 isolates that formed biofilm, genotypic positive, while 26.1% were genotypic negative. This is in agreement with other studies **Kamali et al.** [27], **Banar et al.** [20] and **Ghadaksaz et al.** [29]. The capacity of some strains to form biofilm in the absence of investigated genes shows other genetic variables involved in *P. aeruginosa* biofilm production [31,32]. We believe that biofilm development in *P. aeruginosa* is influenced by a number of variables other than these investigated genes, as these genes were found in both weak and powerful biofilm producing isolates. These variables may be culture conditions (oxygen, temperature, osmolality, pH, nutrients and iron), presence of additional genes involved in biofilm formation, genes expression rate, in addition,

flagella-mediated motility has a role in primary step of biofilm formation [5].

Conclusion

Our results detected high prevalence of MDR pattern among *p. aeruginosa* isolates and significant association between MDR and biofilm formation. Also, prescence of *(algD /pslD /pelf)* genes was found to contribute to high degree in biofilm production.

Conflict of interest: All authors declare no conflict of interest in this work.

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Contributors and authorship

All authors have made substantial contributions to the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content. Finally, they have approved the version to be submitted.

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