Prevalence of Biofilm Genotype Pattern(*algD −/pslD −/pelF* –) with Multidrug-Resistant in Clinical Local *Pseudomonas Aeruginosa* Isolates

Duraid K. AlRawi[1], Huda M. Mahmood[2]

[1]Assist.Lecture, Chief Blood Bank of Ramadi, Anbar Health Department, Ministry of Health and Environment,
[2]Assist.prof .Department of Biotechnology College of Science, University of Anbar

Abstract

The study was designed to explore the distribution and association of the biofilm genotype pattern(*algD −/pslD −/pelF* –) with multidrug-resistant in clinical local *Pseudomonas aeruginosa* isolates. A total of one hundred isolates of *Pseudomonas aeruginosa* were included in this study, which has been collected from different specimens, from July to September 2020. The isolates included were 34 from burns, 19 from wounds, 23 from ear infections, 22 from urinary tract infections (UTI), and 2 from cystic fibrosis (CF). Identification of the isolates was carried out using microscopical, cultural characterization on MacConkey agar, Cetrimide agar, then Pseudomonas agar. Biochemical tests were performed, and further identification was carried out by the VITEK_2_compact system. Genotypic identification has been completed *by16SrRNA*. To assess the frequency of multidrug-resistant of *Pseudomonas aeruginosa* (MDR), the antibiotic susceptibility test was done. It was carried out by using different groups of antibiotics (10 antibiotics) using the Kirby–Bauer disk diffusion method. The results showed that the resistance were Ceftazidime(62%),Gentamicin(26%,(Piperacillin-tazobactam(25%), Ticarcillin(24%), Meropenem(20%),Cefepime(18%),Amikacin(17%) Levofloxacin(16%),Colistin(15%) Imipenem(10%). Biofilm production was assessed using a microplate examination method. The results showed that 93% of isolates were positive for biofilm production, while (7%) were non-biofilm producers. There were differences in the rates of biofilm-production distributed into 21 (21%) were strong biofilm producer (OD was more than 2.156), 25 (25%) intermediate biofilm producer, and 47 (47%) were weak biofilm producer (OD was less than 1.078), and the non -biofilm producer was 7(7%).

Three virulence factors genes (*algD*, *pslD*, and *pelf*) were chosen, which responsible for the phenotypic pattern of biofilm formation and identified as genotypic *algD −/pslD −/pelF* – pattern. The differences in genotypic pattern prevalence among the MDR-positive isolates of different origins were statistically significant. Chi-square analysis showed a highly significant association between strong biofilm capacity and genotype pattern ($p<0.0001$), also the analysis showed a highly significant association between moderate biofilm capacity and genotype pattern ($p<0.002$). Chi-square analysis showed a highly significant association between weak biofilm capacity and genotype pattern ($p<0.001$).

In the current study the percentage of resistance among *P. aeruginosa* local isolates for multiple antibiotics (MDR) was relatively low, maybe due to the combination strategies based on appropriate anti-pseudo-antibiotic agents that may be used to improve treatment from the related infections, according to these results, *P. aeruginosa* local isolates that produced biofilm were mostly (70%) indicated as non-MDR.

Key words: *Biofilm formation, MDR,* **Pseudomonas aeruginosa,** *genotype pattern*

Corresponding author:
Huda M. Mahmood
huda.mahmood@uoanbar.edu.iq

Introduction

Pseudomonas aeruginosa, the primary human pathogen in the *Pseudomonas* genus, it is an important opportunistic bacterium and a leading cause of nosocomial hospital-acquired infections. *P. aeruginosa* is a regular cause of nosocomial pneumonia, urinary tract infections(UTI), it may also colonize in healthy humans without causing the disease [1]. *Pseudomonas. aeruginosa* is a motile, aerobic Gram-negative rod bacterium located in a range of biotic or abiotic habitats, such as soil, water, animals, plants, and insects [2]. *Pseudomonas* species are aerobic, non-spore-forming, straight, or slightly curved gram-negative rods of 0.5–1.0μm by 1.5–5.0μm. They're motile with one or more polar flagella, very strict oxygen aerobic respiratory metabolism, but in certain cases, nitrate has been used as an alternative that allows anaerobic growth[3]. It can produce a variety of pigments, such as pyocyanin (blue-green) and fluorescein (yellow-green), and also the capacity of some strains to produce other pigments, like yellow pyoverdin, dark red pyorubin, and pyomelanin (dark black) [4]. It has great genomic content (~6.5 Mbp) for variations in metabolism and adaptation for several environmental roles and mismatch repair systems [5].

Multidrug-resistant *P. aeruginosa* (MDR-PA) is one of the great concerns as it does not only cause fatal and serious infections, it increases the length of stay in the hospital, leading to increased treatment costs[6]. The main types of antibiotic resistance developed are natural (Intensive) resistance, acquired resistance, cross-resistance, and multidrug resistance as well as pan-resistance[7]. Once biofilms are recognized as the source of disease, management becomes very problematic. Typically, instant controller by extra of high -dose for antibiotic required for long term administration. Biofilm is formed of surface-adjusted aggregates of bacteria integrated with self-made extracellular polymeric substances(EPS), which decrease the probability that the bacteria will penetrate the immune cells as well as antibiotics within the biofilm and serves as a useful defense against the host immune system and antibacterial compounds, resulting in continuous colonization leading to treatment failure[8].

The biofilm components of *Pseudomonas aeruginosa* consist of three distinct exopolysaccharides, which include alginate, Psl, and Pel [10]. Alginate is a polymer made up of -D-mannuronic acid and -L-guluronic acid that contributes significantly to the structural support and protection of biofilms. Psl is a polysaccharide made up of repeated pentasaccharides, made up of D-mannose, D-glucose, and rhamnose Psl is necessary for biofilm development and for the biofilm structure to be protected. Pel is the third polysaccharide found in *Pseudomonas aeruginosa* biofilm and is high in glucose[60]. Different virulence factors such as lipopolysaccharide, flagellum, type IV pili, type III secretion system, proteases, alginate, exotoxin A, quorum sensing(QS), biofilm formation, type VI secretion systems, and oxidant generation in the airspace, may be cited as pathogenicity, and they affect in various ways on the immune response [11]. Quorum Sensing is a communication method that bacteria use to regulate the density of the population by producing as well as sensing small diffuse signal molecules. This form of intercellular bacterial signaling coordinates gene regulation and controls several cooperative behaviors, which include biofilm formation, virulence traits, metabolic demands, and host-microbe interactions [12]. This study aims were: isolation and characterization of *Pseudomonas aeruginosa* from different clinical specimens, also screening of the multidrug-resistant, and biofilm formation isolates, evaluate the phenotypic and genotypic characteristics of biofilm production rates, and the association between resistance patterns and their biofilm capacity.

Materials and Methods

One hundred samples from Anbar Governorate were randomly included in the study. These were obtained from patients admitted to the Urology and Dermatology Departments in Al-Ramadi Teaching

Hospital and Burn department. All the samples were collected during the period from July to December 2020. The study isolates were obtained from urinary tract infections (UTI), wound infections, including burns, otitis media, and other types of wounds, out of these isolates, 100(100%) isolates were identified as *P. aeruginosa*., 93(93%) isolates were produced biofilm.

All isolates were identified as *P. aeruginosa* according to morphological, cultural, biochemical characteristics, VITEK-2 and *16S rRNA* proposed by [13, 14].

Antimicrobial Susceptibility Test(AST):

Depending on the definition of the Clinical and Laboratory Standards Institute (CLSI-2019) using the agar diffusion method. All these isolates were tested for as well as (*Pseudo* ATCC:15442) was used as a standard strain**.**

Quantification of biofilm production

Microtiter plate assay:

Microtiter plate assay was achieved according to [15]. The results were compared according to the following equations (Table 1).

Table (1): The results were calculated based on the following equation:

Mean OD630	Biofilm Intense
OD≤ ODC*	Non-producer
ODC<OD≤ 2ODC	Weak
ODC<OD≤ 4ODC	intermediate
OD>4ODC	Strong

***Cut off value (ODC)=Mean OD of negative Control +3 (Standard Deviation of control).**

Methods of PCR for detection of specific genes

1 Primers Solutions:

The primers were established based on the National Center for Biotechnology Information NCBI and provided by the Promega Company as a lyophilized product of various concentration of picomol (Table 2).. Solution final concentration of (10 pmol/µl) was prepared separately by dissolving 10µl of stock solution for each primer and added to 90µl free nuclease distilled water un-ionic(ddH_2O), mixed well and kept in (-20^oC). They were mixed by vortex to homogenize before use.

Table (2): Sequence of PCR primer and molecular size of PCR products.

Gene		Sequence of forward and reverse (primer 3/-5/)	TM(C0)	Product (bp)	Reference
16srRNA	F	AGAGTTTGATCCTGGCTCAG	58	1500	[16]
	R	CTACGGCTACCTTGTTACGA			
algD	F	CTACATCGAGACCGTCTGCC	58	593	[17]
	R	CATCAACGAACCGAGCATC			

Cont... **Table (2): Sequence of PCR primer and molecular size of PCR products.**

PslD	F	TGTACACCGTGCTCAACGAC	56	369	[17]
	R	CTTCCGGCCCGATCTTCATC			
PelF	F	GAGGTCAGCTACATCCGTCG	58	789	[17]
	R	TCATGCAATCTCCGTGGCTT			

F=Forward sequence, R=Reverse sequence.

PCR program for *16SrRNA, algD* and *pelF and pslD* genes detection:

PCR was used for the detection of *Pseudomonas aeruginosa*. PCR tubes containing The mixture were transferred to the preheated thermocycler and began the program as shown in the tables below (Table 3,4 and 5).

Table (3): PCR amplification program for*16SrRNA* detection.

Step	Temp.(C0)	Time	NO. of cycle
Initial denaturation	95	5min	1
denaturation	95	1 min	35
Annealing	58	40 second	30
extension	72	45 second	
Final extension	72	5 min	1
Hold Temperature	4	3min	-

2.12 Statistical Analysis:

Data analysis was performed using the available statistical package SPSS-22. Data was reflected in simple frequency and percentage measurements. The significance of the difference in different percentages (quality data) was evaluated using the Chi-square test (X^-). Statistical significance was considered whenever the P-value for the relevance check was equal to or less than the P-value for the relevance check (0.05).

Results and Discussion

Isolation and identification of *Pseudomonas aeruginosa*:

The current study has been carried out on 100 clinical samples from including catheter-acquired urine and wound infections and also burn, cystic fibrosis, and otitis media patients from July 2020 to September 2020. Microbial isolates were collected from patients who are admitted to the Urology and Dermatology Wards of the Al-Ramadi Teaching Hospital (Ramadi, Iraq) and the laboratory of Al-Ramadi Teaching Hospital. The isolates were identified by various types of media and chemical characteristics of *Pseudomonas aeruginosa.*

Prevalence of *Pseudomonas. aeruginosa isolates*

After all confirmation tests for the identification

of *Pseudomonas aeruginosa*, a total of one hundred isolates were obtained . The results showed that the highest ratio of *P.aeruginosa* isolates was from burns with percentages of 34(34 %). This may be due to the pathogen has several potentially virulent factors which help it to colonize and infect mammalian tissues, like protease, pyocyanine, and hemolysin, which promote adherence to host cells, destroy host tissue, and disrupt the defense system[18,19]. As a result of the damage to the skin barrier in burn patients and repeated scrub of the burn site with high resistance to topical povidone-iodine as well as the cross-contamination with *Pseudomonas* strains are often more likely to occur[20][21]. Abdullah et al,2019 [22] indicated that the percentage of *P.aeruginosa* from the wound was 14%, and this slightly close to our results.

However, the above results indicated that *P.aeruginosa* is one of the most species that causes wound and burn infections because this pathogen is opportunistic and can cause infection to any deficiency in the body's defense system.[23]. The percentage of other infections were (23%) in the ear, (22 %) in the urine of total clinical isolates. Our study also showed that the ratio of bacteria isolated from the urine was 22 %. Whereas, this result varied from [24], which found that the isolation rate from ear infections was 11.6%. This may be due to the difference in isolation sites, the number of isolates, type of samples, and the distribution of isolates that may vary depending on the location of the infection, and some other reasons, like the type of sterilization and disinfectants used in hospitals besides the methods used in sterilization. Since this pathogen is resistant to many sterilizers and antimicrobial agents(Fgure 1) [25].

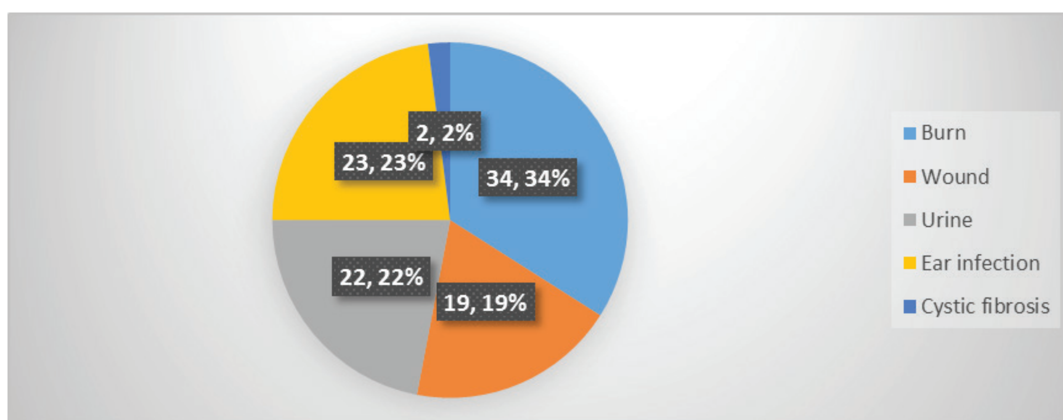

Figure (1): The source of the specimens and their number and percentage (%).

Kirby Bauer Disk Distribution Susceptibility Results:

One hundred *Pseudomonas aeruginosa* isolates from the burn, wound, urine, ear, and CF were tested for antibiotic sensitivity by the Kirby Bauer Disk Diffusion method as recommended by (CLSI, 2019). The antigram of the isolates studied showed variable resistance toward most of the antibiotics under study as shown in figure (2). Susceptibility was tested for 10 antimicrobials agents including Ceftazidime(CAZ),Imipenem(IPM),Piperacillin-tazobactam(PRL),Levofloxacin(LEV), Meropenem (MEM), Gentamicin(GN), Colistin(CO), Amikacin (AK), Ticarcillin(TC), Cefepime(FEP/CPM). The isolates were resistant to all antibiotics used in our study. An increase in bacterial resistance to many antibiotics considered to be a major therapeutic challenge. The results showed that the highest resistant percentage was 62% to Ceftazidime and the lowest percentage was 10% to Imipenem antibiotic.

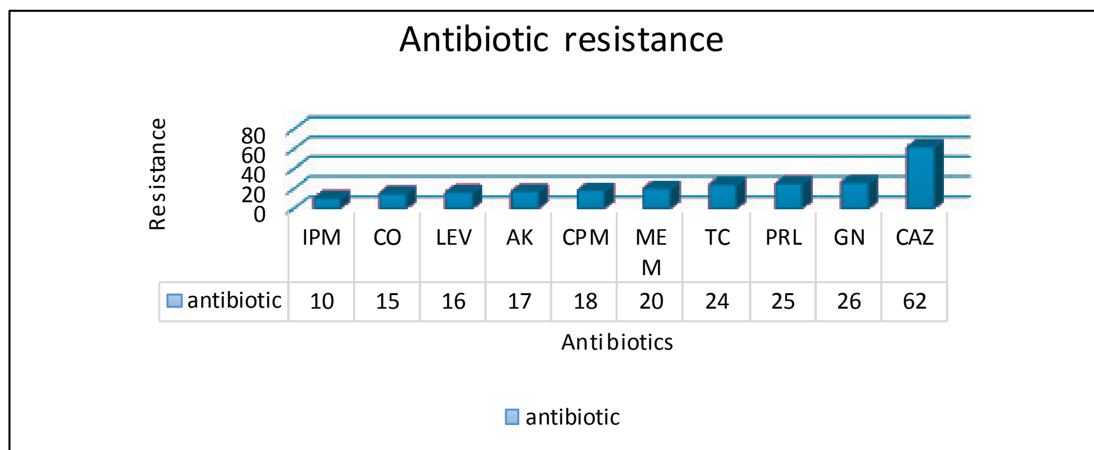

Figure (2): Antibiotic Resistance ratios among *P. aeruginosa* isolates. Ceftazidime(CAZ),Imipenem(IPM), Piperacillin-tazobactam (PRL), Levofloxacin(LEV), Meropenem(MEM), Gentamicin(GN), Colictin(CO), Amikacine(AK), Ticarcilin(TC), Cefepime (FEP/CPM).

The present study showed a different percentage of *P.aeruginosa* resistance to ceftazidime in comparison to some previous studies, and this percentage of *P.aeruginosa* resistance to ceftazidime was lower than the result achieved in the study by [26], and higher than[27],[28].

Biofilm estimation by microtiter plate assay

The results of the quantifiable biofilm formation assay showed that the various *P. aeruginosa* biofilm producers were classified as strong, moderate, and weak. In the qualitative biofilm formation assay, a spectrophotometric technique was used under a set of experimental situations. Our results showed 100 isolates were *P. aeruginosa*, 93% were biofilm producers, distributed into 21% strong biofilm producer (OD was more than 2.156), 25% intermediate biofilm producer, and 47% were weak biofilm producer (OD was less than 1.078), and the non -biofilm producer was 7%.

Isolates from the wound were 19% which distributed into 9.52 %, 12 %, and 29.78 %, strong, intermediate, and weak biofilm producers respectively. 19% isolates were collected from UTI out of them, 19% isolates were biofilm producers. 23.40% isolates were weak biofilm producers, 20% were intermediate, 14.28% isolates were strong biofilm producers. Non -biofilm producer was 42.85%. Out of 100isolates of *P. aeruginosa*, 23% were collected from ear infections, 19% isolates, 23 isolates were biofilm producers. Distributed into 27.65% isolates were weak biofilm producers, 20% were intermediate, 14.28% isolates were strong biofilm producers, and the non –biofilm producers were 2%. Two percent were collected from CF, and they were biofilm producers. Out of these 2 isolates, 4.25% isolates were weak biofilm producers. However, the TCPM is considered as the standard phenotypic test for the assessment of biofilm formation and it was the most specific test in the current study. It was also an easy test in the laboratory to detected biofilm formation in quantitative ways. Furthermore, the verification of the TCPM results is accomplished by using an ELISA reader which considerably decreases the subjective errors shown with other phenotypic tests.

In the present study, the TCPM strong biofilm formation in 21% of isolates. These results were higher than those by Panda et al, 2016 [29] who reported 11.00%. In a study performed by Jabalameli et al, 2012 [30], biofilm production has been observed

in more than 96% of the isolates which 22.9% were weak biofilm formers, 26% were moderate, and 47% were strong. The results of this study showed tht the biofilm producers have been observed in more than 93% of the isolates , which werer 25% moderate, while this study did not agree with our results 21% of strong biofilm and weak biofilm 47%.

Molecular Identification of the local isolates

Using housekeeping genes in the molecular analysis (e.i *16S rRNA*) led to the advancement toward rapid techniques diagnostic for the identification *of P. aeruginosa* isolates [31]. The PCR technique is a highly sensitive and fast tool utilized for bacterial detection is that highly conserved and unable to change over time, and provides a specific sequence to each type [32].

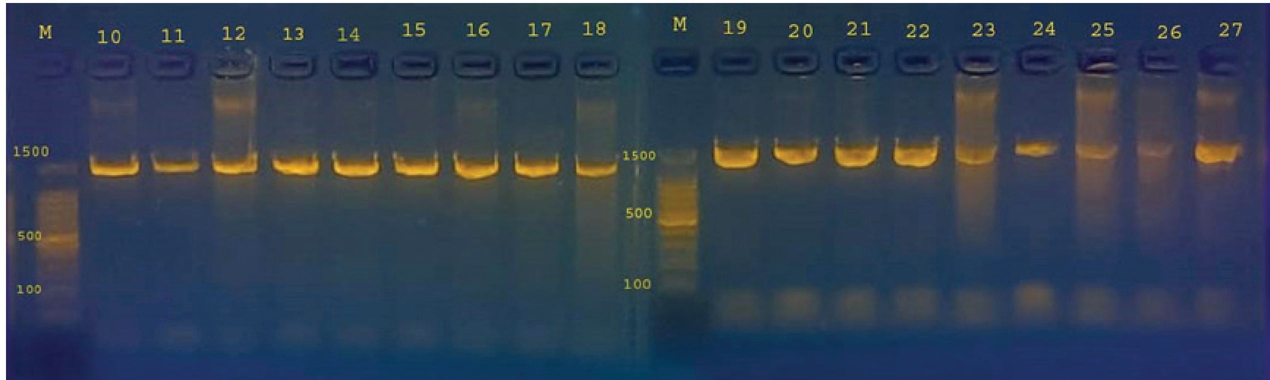

Figure (3): Agarose gel electrophoresis (1.5%agarose, 7 V/cm^{2}for 90min) of *16srRNA* gene (1500bp). Lane M 1500bp DNA ladder, Lanes10-27 represent bands of *P.aeruginosa* isolates, stained with ethidium bromide and visualized on a UV trans illuminator.

In this study, all isolates are diagnosed genetically by using a PCR technique depending on the *16S rRNA* gene. Figure 6 showsed agarose gel electrophoresis (90 min with 1.5% agarose) for *16S rRNA* with PCR products (amplified size 556 bp). All of the isolates 51/51(100%) gave positive results during this genotyping test, where its bands appear at the same level in the agarose gel, this result matched with several studies such as [33][34]. Since the genetic diagnosis results of the *16S ribosomal RNA* were conforming to the morphological and biochemical test results; all bacterial isolates that were subjected to diagnosis were confirmed as *P. aeruginosa* isolates (Figure 3).

Relationship between biofilm characteristic and genotype patterns among *P. aeruginosa* clinical isolates

Biofilm phenotypes accounted for 93 % (n = 93) out of 100 isolates , being distributed in the following groups: 21% (n = 21) produced strong biofilm; 25% (n = 25) produced moderate biofilm; 47% (n = 47) produced weak biofilm, whilst 7% of isolates (n = 7) were identified as non-biofilm producer (Table 6). A high existence of biofilm-encoding genes were found, 73.89% (n = 17) of the isolates presented all three *algD*, *pslD*, and *pelF* genes, at the same time (considered as *algD* +/*pslD* +/*pelF* + genotypic pattern), 17.38% (n=4) presented only two gene *algD*, *pslD* (considered as *algD* +/*pslD* +/*pelF* - genotypic pattern), 4.34%(n=1) showed only one gene *algD* +(considered as *algD* +/*pslD* -/*pelF* -), while 4.34% (n =1) had none of the three genes and identified as *algD* −/*pslD* −/*pelF* − pattern. The present study revealed a high prevalence of *algD*, *pslD*, and *pelF* genes, being presented simultaneously in a considerable percentage (73.89%) of *P. aeruginosa* isolates, the result is similar to those found by [35]. Other genes

related to biofilm formation, such as *pslA* and *pelA* were noticed by Ghadaksaz et al [36].with a rate of 83.7% and 45.2%, respectively, and pournajaf et al[37], with a rate of 89.5% and 57.3%, respectively within *P. aeruginosa* clinical isolates. However, minute data is available about the frequency rate of *pslD* and *pelF* genes in different areas of the world.

Chi-square analysis showed a highly significant association between strong biofilm capacity and genotype pattern (X^2= 25, d.f=1, p<0.0001), also the analysis showed a highly significant association between moderate biofilm capacity and genotype pattern (X^2= 12.42, d.f=2, p<0.002), and Chi-square analysis showed a highly significant association between weak biofilm capacity and genotype pattern (x^2= 13.77, d.f=2, p<0.001)(Table 6).

Table (6): Relationship between biofilm characteristic and genotype patterns among *P. aeruginosa* clinical isolates.

Genotypic biofilm pattern, No. (%)	Phenotypic biofilm pattern, No. (%)		
	Strong	Moderate	Weak
algD +/pslD +/pelF +	2(8.69)	5(21.73)	10(43.47)
algD +pslD +/pelF −	0(0)	2(8.69)	2(8.69)
algD +/pslD -/pelF -	0(0)	1(4.34)	0(0)
algD -/pslD -/pelF -	0(0)	0(0)	1(4.34)
P-value	0.0001	0.002	0.001
Total	2(8.695)	8(34.782)	13(56.521)

In agreement with other studies byBanar, Ghadaksaz, and Kamali [17][36] [10][, which have shown a significant association between the biofilm-forming ability and the existence of related genes (p-value < 0.0001). The ability of biofilm production despite the absence of biofilm genes studied indicates other genetic determining factors of biofilm contribute to matrix development in *P. aeruginosa*[38,39,40]. By similarity, the absence of biofilm production with the existence of genes may be due to chromosomal mutations in diverse regulatory and controlling systems, influence the production of efficient biofilm-related proteins. Other researchers reported that 31.03% of *P. aeruginosa* isolates contained the *pslA* gene and none of them were phenotypically positive for biofilm production in Congo red agar and microtiter plate assays[41]. Lima et al and Hou et al [42]reported that the mutations in *lasI/lasR* and *rhlI/rhlR* systems lead to phenotypical changes in quorum sensing proteins as the reason why these isolates are unable to produce biofilm[41][42] While, in another study, Abidi et al [43] reported that biofilm production was significantly higher in MDR isolates. In a study by Bogiel et al [44] on the *algD* gene's existence, 99 (92.5%) CRPA isolates were positive, these results are consistent with the results of the research carried out by Ellappan et al [45] showing that 92.9%. According to the findings of the current study, we can conclude:

the highest percentage of *P. aeruginosa* antibiotic resistance was 62% towards Ceftazidime(CAZ) and the lowest was 10% against Imipenem(IPM), most isolates of *P. aeruginosa* were the highest production of biofilm (93%). while few isolates were non-producer (7%), and there is a variation of genotypic patterns of biofilm production capacity *algD, pelFand pslD* in MDR local *P. aeruginosa* clinical isolates, the results showed that most biofilm producers were mainly considered as non-MDR.

Funding : The authors received no financial support for the research, authorship, and/or publication of this article

Conflict of Interest : there has been no conflict of interest of any kind with the authors of this work.

Ethical Standard: The study was formally approved the research plan by the ethical committee board at the Anbar health directorate.

Informed Consent was taken from all the participant patients before being enrolled in the study. **All data and materials are available**

References

1. Lila G, Mulliqi-Osmani G, Bajrami R, Kurti A, Azizi E, Raka L. The prevalence and resistance patterns of *Pseudomonas aeruginosa* in a tertiary care hospital in Kosovo. Infez Med. 2017;25(1):21–6.
2. Al-Wrafy F, Brzozowska E, Górska S, Gamian A. Pathogenic factors of *Pseudomonas aeruginosa* - the role of biofilm in pathogenicity and as a target for phage therapy. Postepy Hig Med Dosw. 2017;71(February):78–91.
3. Diggle SP, Whiteley M. Microbe profile: *Pseudomonas aeruginosa*: Opportunistic pathogen and lab rat. Microbiol (United Kingdom). 2020;166(1):30–3.
4. Sameet MC, Awadh HA, Suleiman AAJ. Effect of Isolation Source on Virulence Gene Expression, Phenotypic and Antibiotic Resistance Patterns of Clinical Isolate of *Pseudomonas aeruginosa*. Ann Trop Med Public Heal. 2020;23(1).
5. Mahmood, H.M. G.A.Nasir, Q.A.Ibraheem. Relationship between pigments production and biofilm formation from local *Pseudomonas aeruginosa* isolates. *IJAS*.2020; 51(5):1413-9.
6. Ming-Wun W, Chih-Hsum Y, Tso-Tsai L, Wei-Yi L, Jui-Sheng H, Chin-Lon L, et al. Testing for weak D Antigen: Spectrum and its applied role in rhesus-negative transfusions in Andaman and Nicobar Islands Rupinder Kaur Brara*, P. S. Shaijib, Sahil Sehgalc. Vol. 30, Tzu Chi Medical Journal. 2018. 200–203 p.
7. Cesur S, Demiröz AP. Antibiotics and the Mechanisms of Resistance to Antibiotics. Med J Islam World Acad Sci. 2013;21(4):138–42.
8. Iman Abbas AL-Essawi, Huda M. Mahmood. Green synthesis of gold nanoparticles and their effect on pyocyanin pigment production from local *Pseudomonas aeruginosa* Isolates. *Annals of RSCB*. 2021. 25(1):6737-48.
9. Gebreyohannes G, Nyerere A, Bii C, Sbhatu DB. Challenges of intervention, treatment, and antibiotic resistance of biofilm-forming microorganisms. Heliyon [Internet]. 2019;5(8):e02192. Available from: https://doi.org/10.1016/j.heliyon.2019.e02192.
10. Kamali E, Jamali A, Ardebili A, Ezadi F, Mohebbi A. Evaluation of antimicrobial resistance, biofilm forming potential, and the presence of biofilm-related genes among clinical isolates of *Pseudomonas aeruginosa*. BMC Res Notes [Internet]. 2020;13(1):4–9. Available from: https://doi.org/10.1186/s13104-020-4890-z
11. Rocha AJ, De Oliveira Barsottini MR, Rocha RR, Laurindo MV, De Moraes FLL, Da Rocha SL. *Pseudomonas aeruginosa*: Virulence factors and antibiotic resistance Genes. Brazilian Arch Biol Technol. 2019;62:1–15.
12. Turkina M V., Vikström E. Bacteria-Host Crosstalk: Sensing of the Quorum in the Context

of *Pseudomonas aeruginosa* Infections. J Innate Immun. 2019;11(3):263–79.

13. Overhage J, Schemionek M, Webb JS, Rehm BHA. Expression of the psl operon in *Pseudomonas aeruginosa* PAO1 biofilms: PslA performs an essential function in biofilm formation. Appl Environ Microbiol. 2005;71(8):4407–13.
14. Klainer AS. Use of gram stain in clinical infectious diseases. Schering; 1976.
15. Bose S, Khodke M, Basak S, Mallick SK. Detection of biofilm producing staphylococci: Need of the hour. J Clin Diagnostic Res. 2009;3(6):1915–20.
16. Amoon RH, Abdallha AH, Sharif AO, Moglad EH, Altyb HN, Elzaki SG, et al. Molecular characterization of *Pseudomonas aeruginosa* isolates from Sudanese patients: A cross-sectional study. F1000Research. 2018;7(July).
17. Banar M, Emaneini M, Satarzadeh M, Abdellahi N, Beigverdi R, Van Leeuwen WB, et al. Evaluation of mannosidase and trypsin enzymes effects on biofilm production of *Pseudomonas aeruginosa* isolated from burn wound infections. PLoS One. 2016;11(10):1–13.
18. Da Silva N, Taniwaki MH, Junqueira VCA, Silveira N, Okazaki MM, Gomes RAR. Microbiological examination methods of food and water: a laboratory manual. CRC Press; 2018.
19. Humphries RM, Linscott AJ. Laboratory diagnosis of bacterial gastroenteritis. Clin Microbiol Rev. 2015;28(1):3–31.
20. Japoni A, Farshad S, Alborzi A. *Pseudomonas aeruginosa*: Burn infection, treatment and antibacterial resistance. Iran Red Crescent Med J. 2009;11(3):244–53.
21. Coetzee E, Rode H, Kahn D. *Pseudomonas aeruginosa* burn wound infection in a dedicated paediatric burns unit. South African J Surg. 2013;51(2):50–3.
22. Abdullah R, Falih A. Genotyping of *Pseudomonas aeruginosa* Isolated from Different Clinical Samples by Using ERIC Method. Diyala J Pure Sci. 2019;15(2):107–17.
23. Goering R, Dockrell H, Zuckerman M, Chiodini PL. Mims' Medical Microbiology E-Book. Elsevier Health Sciences; 2018.
24. Hamid AN, Maeny SAL Al, Nader MI. Relationship of Lectins *Pseudomonas aeruginosa* bacteria with some other virulence factors. 2017;1:108–18.
25. Kiffer C, Hsiung A, Oplustil C, Sampaio J, Sakagami E, Turner P, et al. Antimicrobial susceptibility of gram-negative bacteria in Brazilian hospitals: The MYSTIC Program Brazil 2003. Brazilian J Infect Dis. 2005;9(3):216–24.
26. Rahman Rasheed Al-Taai H, Mohammed Hameed Z, Mohammed Jasim I. Investigation on bla Ctx-M-1 , Virulence Factors and Multidruge Resistance in *Pseudomonas aeruginosa* Bacteria. Diyala J Pure Sci. 2017;13(3):206–21.
27. Raoof WM, Tawfiq ALSM. Sensitivity of *Pseudomonas aeroginosa* Isolated from patients with different clinical state to Antibiotics and determination of some virulance factors in vitro. Tikrit J Pure Sci. 2015;20(1).
28. Razzaq AHA, Hussain AS, Omar AA. Study the synergistic effect of Alcoholic extract of Capparis spinose and Iraqi Dates vinegar wolution with Antibiotics on Isolates of *Pseudomonas aerugenosa*. 2017;1:84–93.
29. Panda PS, Chaudhary U, Dube SK. Comparison of four different methods for detection of biofilm formation by uropathogens. Indian J Pathol Microbiol. 2016;59(2):177.
30. Jabalameli F, Mirsalehian A, Khoramian B, Aligholi M, Khoramrooz SS, Asadollahi P, et al. Evaluation of biofilm production and characterization of genes encoding type III secretion system among *Pseudomonas aeruginosa* isolated from burn patients. Burns. 2012;38(8):1192–7.

31. Srinivasan R, Karaoz U, Volegova M, MacKichan J, Kato-Maeda M, Miller S, et al. Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. PLoS One. 2015;10(2):1–22.

32. Abdullah RM, Mehdi AF. Identification of *Pseudomonas aeruginosa* From Clinical Specimen by Using 16S rDNA Gene. J Biotechnol Res Cent. 2016;10(1).

33. Jaafar ZM, Dhahi MAR, Abd AKH, Jaafar SM. Molecular identification and antibiotics resistance genes profile of *Pseudomonas aeruginosa* isolated from Iraqi patients. African J Microbiol Res. 2014;8(21):2183–92.

34. Crovadore J, Grizard D, Chablais R, Cochard B, Blanc P, Lefort F. Whole-genome sequence of *Pseudomonas aeruginosa* Strain 4014, isolated from soil in France. Microbiol Resour Announc. 2018;7(10).

35. Banar M, Emaneini M, Satarzadeh M, Abdellahi N, Beigverdi R, Van Leeuwen WB, et al. Evaluation of mannosidase and trypsin enzymes effects on biofilm production of *Pseudomonas aeruginosa* isolated from burn wound infections. PLoS One. 2016;11(10):1–13.

36. Ghadaksaz A, Imani Fooladi AA, Hosseini HM, Amin M. The prevalence of some *Pseudomonas* virulence genes related to biofilm formation and alginate production among clinical isolates. J Appl Biomed [Internet]. 2015;13(1):61–8. Available from: https://doi.org/10.1016/j.jab.2014.05.002

37. Pournajaf A, Razavi S, Irajian G, Ardebili A, Erfani Y, Solgi S, et al. Integron types, antimicrobial resistance genes, virulence gene profile, alginate production and biofilm formation in Iranian cystic fibrosis *Pseudomonas aeruginosa* isolates. Infez Med. 2018;26(3):226–36.

38. Friedman L, Kolter R. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. Mol Microbiol. 2004;51(3):675–90.

39. Müsken M, Di Fiore S, Dötsch A, Fischer R, Häussler S. Genetic determinants of *Pseudomonas aeruginosa* biofilm establishment. Microbiology. 2010;156(2):431–41.

40. Moradali MF, Ghods S, Rehm BHA. *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. Front Cell Infect Microbiol. 2017;7:39.

41. Hou W, Sun X, Wang Z, Zhang Y. Biofilm-forming capacity of *Staphylococcus epidermidis, Staphylococcus aureus,* and *Pseudomonas aeruginosa* from ocular infections. Invest Ophthalmol Vis Sci. 2012;53(9):5624–31.

42. da Costa Lima JL, Alves LR, de Araujo Jacome PRL, Neto JPB, Maciel MAV, de Morais MMC. Biofilm production by clinical isolates of *Pseudomonas aeruginosa* and structural changes in LasR protein of isolates non biofilm-producing. Brazilian J Infect Dis. 2018;22(2):129–36.

43. Abidi SH, Sherwani SK, Siddiqui TR, Bashir A, Kazmi SU. Drug resistance profile and biofilm forming potential of *Pseudomonas aeruginosa* isolated from contact lenses in Karachi-Pakistan. BMC Ophthalmol. 2013;13(1):1–6.

44. Bogiel T, Depka D, Rzepka M, Kwiecińska-Piróg J, Gospodarek-Komkowska E. Prevalence of the Genes Associated with Biofilm and Toxins Synthesis amongst the *Pseudomonas aeruginosa* Clinical Strains. Antibiotics. 2021;10(3):241.

45. Ellappan K, Narasimha HB, Kumar S. Coexistence of multidrug resistance mechanisms and virulence genes in carbapenem-resistant *Pseudomonas aeruginosa* strains from a tertiary care hospital in South India. J Glob Antimicrob Resist. 2018;12:37–43.