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DETECTION OF BIOFILM PRODUCTION AMONG MBLS-PRODUCING PSEUDOMONAS AERUGINOSA CARRYING ALGD, PELF, PSLD GENES ISOLATED FROM BURN CENTER IN AL-NAJAF GOVERNORATE, IRAQ

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Abstract:

Background: Pseudomonas aeruginosa is a worldwide cause of chronic and acute infections of burn wounds. Aim of study: Detection of algD, pelF, and pslD genes encoding biofilm formation among isolates of Pseudomonas aeruginosa that producing metallo β-lactamases. Materials and Methods: 93 isolates of Pseudomonas aeruginosa were isolated from 362 inpatients with infected burn wounds at the burn center in Al-Najaf Governorate, Iraq, from September 2022 to February 2023. Results: Pseudomonas aeruginosa isolates were identified microscopically, phenotypically, culturally, and biochemically then by using the double-disc synergy method to test their capacity to produce metallo β-lactamases. Out of the 25 MBLs-producers, 22 (88%) were biofilm-producing isolates when tested by tissue culture plate method, 4 (16%), 10 (40%), and 8 (32%) were, respectively, weak, medium, and strongly biofilm producers. 25 metallo β-lactamases-producers under study, which had previously been evaluated using biofilm phenotypic method, were subjected to the investigation of biofilm encoding genes by using polymerase chain reaction technique. This molecular technique detected 21 (84%) isolates carried single and multiple biofilm encoding genes, 10(40%) isolates had algD gene, 6 (24%) isolates harbored algD and pslD genes and 5(20%) isolates carried algD, pelF and pslD genes. The single pslD and pelF genes were not detected among test isolates. Conclusions: There was a high prevalence of biofilm-producers among metallo β-lactamases-producing P. aeruginosa isolates. In addition, a clear positive correlation was observed between the multiple genes encoding biofilm and the degree of biofilm production among metallo β-lactamases-producers and close association between metallo β-lactamases and biofilms production, which explains the prevalence of carbapenems resistance and biofilm-production in this study because biofilm provides the appropriate environment for antibiotic resistance in general and carbapenems in particular.

Keywords: *Pseudomonas Aeruginosa, Mbls, Biofilms-Production* .

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1 INTRODUCTION

Pseudomonas aeruginosa is common pathogens in the hospital environment, accountable for 50% of all acute and chronic hospital-acquired infections. The mortality rates that are caused by *P. aeruginosa* are rather significant, ranging from 20–60% [1]. One of the most crucial virulence determinants in this pathogen is the production of biofilms, because of its biofilm-producing potential, using it as an *invitro* model to investigate biofilm formation presents an interesting challenge. Additionally, it colonizes a variety of surfaces, such as medical materials and equipment [2]. Biofilms are cells that are able to aggregate densely and produce components of matrix responsible for maintaining the community's cohesiveness. The manner of biofilm formation enables bacterial cells to remain near to resources, encourages genetic material interchange, and shields cells from various environmental and chemical challenges. Antibiotic resistance in biofilms is around 10-1000 times greater than that in planktonic cells. This is due to proteins synthesis and modification of the metabolic activities as well as the difficulty in penetration of antibiotics into the complex matrix [3]. A biofilm is made up of a matrix that is secreted by the biofilm itself. This matrix is made up of water, RNA, polysaccharides, DNA, and proteins [2].

Exopolysaccharides such as alginate (alg), pellicle (Pel), and polysaccharides synthesis loci (Psl) are the primary building components of the biofilm matrix. These polysaccharides perform many biological functions such as protecting the bacterial cell from the effect of antibiotic agents and shielding it from the immune system [4]. Failure to treat infections with antibiotics has become a serious global problem as a result of the rise in antimicrobial resistance and the widespread spread of related diseases. According to reports, *P. aeruginosa* has a high level of intrinsic resistance to a wide spectrum of antibiotic agents including fluoroquinolone, aminoglycoside, and *β*-lactams resulting in significant mortality and morbidity rates. *β*-lactam therapy is the gold standard for treating *P. aeruginosa* infections, but for many multidrug-resistant (MDR) strains, carbapenems are the only safe therapeutic option left [2]. Carbapenems resistance is an emerging problem due to carbapenemases production, especially metallo *β*-lactamase enzymes (MBLs). Synergistic production of MBLs and biofilm contributes to the widespread of MDR-*P. aeruginosa* globally [5]. As a result of the importance of the relationship between infections acquired by strains of *P. aeruginosa* that produce MBLs and biofilms and the rates of both morbidity and mortality for hospitalized burn inpatients, the current study aimed to identify the prevalence of *algD*, *pelF* and *pslD* genes that encode biofilm production among local MBLs-producing *P. aeruginosa* isolates. Because knowing the characteristics of these strains and their virulence allows the health authorities to take the maximum measures of prevention and health care to limit their spread and transmission between inpatients and increase the possibility of their recovery and survival.

2 MATERIALES AND METHODS

2.1 Patients and Study Samples Collection

This study was carried out from (September 2022) to (February 2023). Smear samples of pus, purulent fluids, and infected skin were randomly collected from inpatients with burn wounds at the burn center in Al-Najaf Governorate of Iraq, wound infection swabs were used to get samples from third-degree burn wounds. Wound infection swabs were used to take samples from patients suffering from third and fourth-degree burn wound infections. Several laboratory tests were conducted to identify isolates of *P. aeruginosa* according to McFadden [6].

2.2 Phenotypic Detection of MBLs-Production

2.2.1 Double-Disc Synergy Test (DDST)

All 93 isolates of *P. aeruginosa* under study were subjected to the DDST to detect their ability to produce MBLs. Two imipenem (IMP, 10µg) discs were laid out on an agar Mueller Hinton agar (HiMedia, India) plate streaked with the 0.5 McFarland of bacterial suspension. One of the IMP discs was saturated with 5 µl of a (0.5 M) of EDTA (HiMedia). The inhibition zones surrounding the IMP discs were evaluated in the absence and presence of EDTA [7].

2.3 Detection of Biofilm Production

2.3.1 Phenotypic Methods

2.3.1.1 Tissue Culture Plate (TCP) Method

Isolates of *P. aeruginosa* were inoculated into test tubes containing 10 ml of Tryptic soy broth supplemented with glucose (HiMedia) at concentration 1%. The test tubes were then incubated for 24 hours at 37 °C. At a ratio of 1:100, fresh medium was used to dilute bacterial suspensions. 200 µl of the diluted bacterial suspensions were then loaded into each well of a microtiters plate. Similarly, control isolate was also added into microtiter plate well. After incubation, the wells' contents were removed, and any remaining bacteria were cleaned out by washing the wells 4 times with 200 l of phosphate-buffered saline 30 minutes of incubation followed the addition of 2% sodium acetate to the wells. Wells with fixed biofilms were then stained with crystal violet solution at concentration 0.1%. The wells were cleansed with deionized water after 30 minutes to eliminate any remaining discoloration. After making sure that the wells were completely dried, an ELISA reader was used to measure the biofilm optical density (OD) at a wavelength of 570 nm. Biofilm formation was categorized as weak, intermediate, or strong according on OD values [8].

2.3.2 Molecular Methods

Three primer pairs, specific for each family of biofilm formation, were chosen to amplify fragments of 593 bp (*algD*), 789 bp (*pelF*) and 369 bp (*pslD*). The primer pairs were: *algD*-F-5'CTACATCGAGACCGTCTGCC3'/ R-5'GCATCAACGAAC CGAGCATC3'; *pel*F-F5'GAGGTCAGCTACATCCGTCG3'/ R-5'TCATGCAATC TCCGTGGCTT3'; *psl*D-F-5'TGTACACCGTGCTCAACGAC3'/ R-5'CTTCCGGC CCGATCTTCATC3' [9].

Ethical considerations

The study was approved by the Postgraduate Studies Committee of the College of College of Health and Medical Techniques, Al-Furat Al-Awsat Technical University, Kufa, Iraq. Patients' consent was taken before clinical samples were collected, and medical professionals at the burn center oversaw the collection of study samples.

Statistical Analysis

The statistical software SPSS v20.0, developed by IBM Corporation, Armonk, New York, USA, was used to analyze the data of the current study.

4 RESULTS

4.1 Phenotypic Detection of Biofilm and MBLs-Producers

In this study, among 362 burn wound infection samples, 93 *P. aeruginosa* isolates were detected. DDST was used to assess the test isolates' capacity to generate MBLs. It was found that 25 (27%) isolates were productive of MBLs and 68 (73%) isolates were non-productive. On the other hands, of the 25 MBLs-producers, 22 (88%) were biofilm-producing isolates when tested by TCP method, 4 (16%), 8 (32%), and 10 (40%) were weak, moderate and strong producers of biofilms, respectively [Figure 1] and [Table 1].

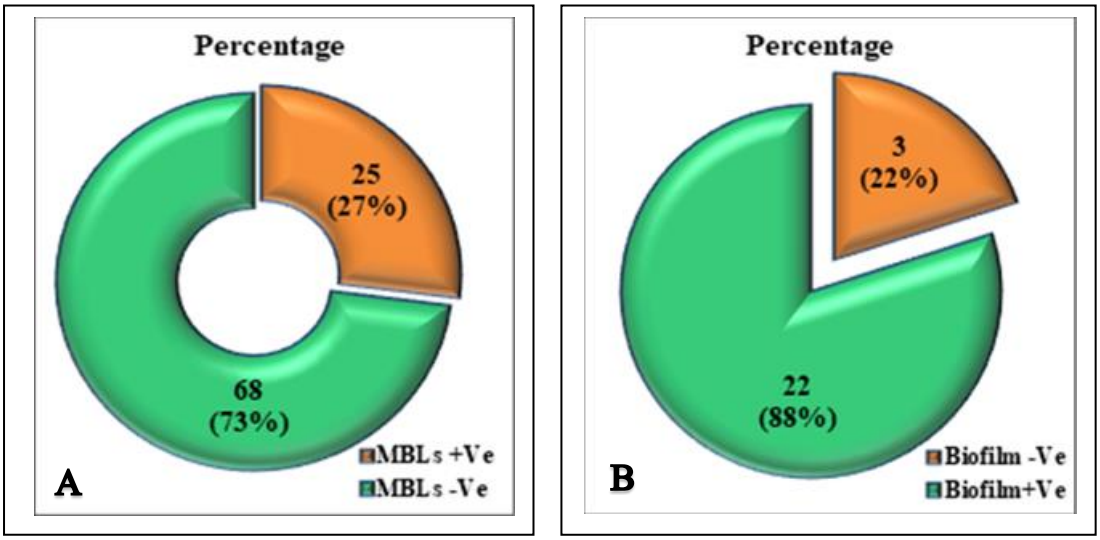

Figure 1. (A), Prevalence of MBLs-producers detected by using DDST; (B), Prevalence of biofilm-formers among MBLs-producers detected by using TCP method.

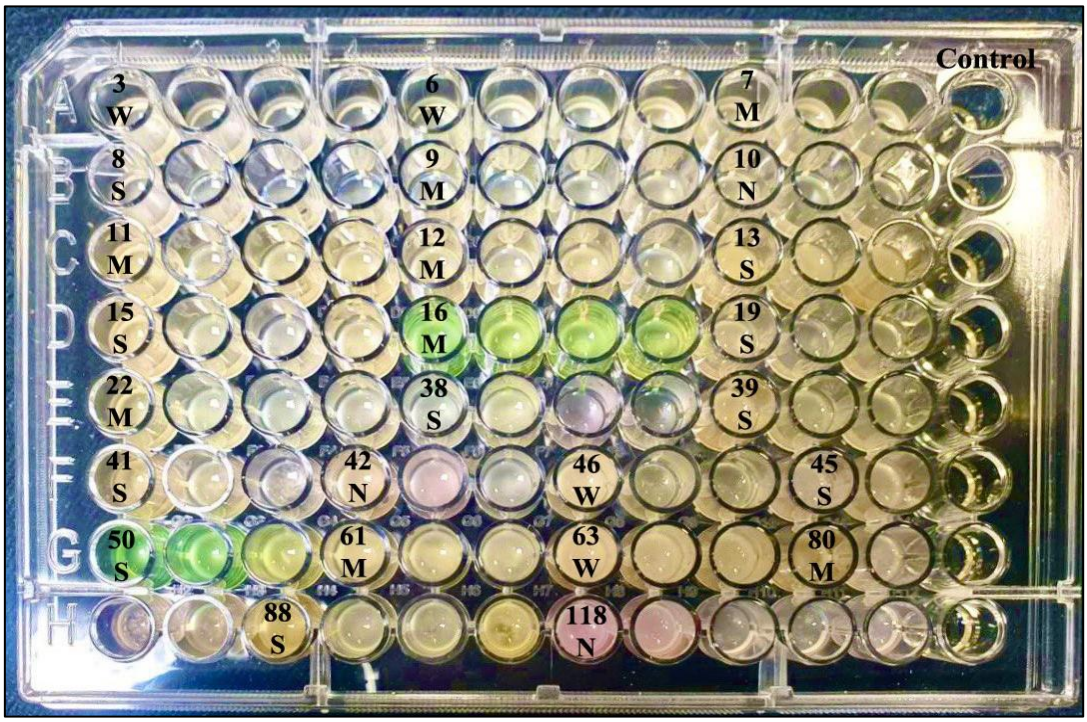

Figure 2. Screening of biofilm production among 25 MBLs producers by using TCP method.

Table 1. Phenotypic detection of biofilm formers among MBLs-producers

Biofilm formers class	No. (%) of biofilm and MBLs-producers n=22/25 (88%)
Strong	10 (40)
Moderate	8 (32)
Weak	4 (16)

4.2 Molecular Detection of Biofilm Formers among MBLs-Producers

The isolates that showed positive results for the DDST were subjected to the standard PCR technique to detect the molecular and genetic profile of the biofilm-producing isolates within the MBLs-producing isolates. Molecular investigation showed that 10 (40%) isolates possessed the *algD* gene and 6 (24%) isolates harbored *algD* and *pslD* genes. Interestingly, there were 5 (20%) isolates that had a distinct molecular profile consisting of *algD*, *pelF* and *pslD* genes [Figure 2] and [Table 2].

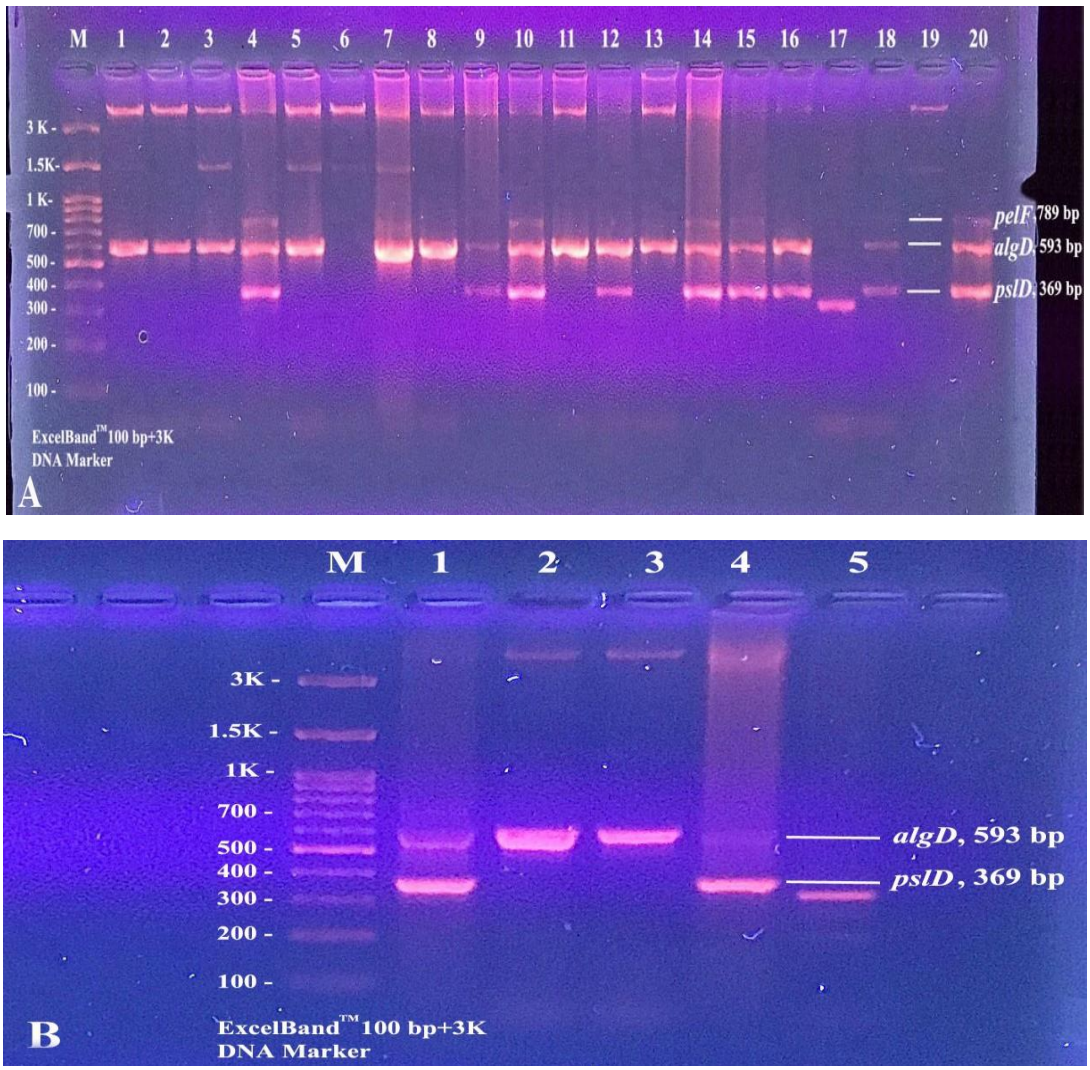

Figure 2. Gel electrophoresis of amplified biofilm genes of MBLs-producers by PCR technique. Electrophoresis was used to separate the PCR products on an agarose gel weighing 0.750 g. For 1.5 hours, 70 Volts were used to conduct the electrophoresis. (A), Lane: M, Marker; lane: 6, 17 and 19 biofilm genes negative; the other lane: biofilm genes positive; (B), Lane: M, Marker; lane: 5 biofilm genes negative; the other lane: biofilm genes positive.

Table 2. Frequency of biofilm encoding genes among 25 of MBLs-producers

Biofilm encoding genes type	No. of biofilm encoding genes N= 21/25 (84%) n (%)	Type of biofilm encoding genes association	N= 21/25 (84%) n (%)
algD	10(40)	Single	10 (40)
pelF	0 (0.0)	-	
pslD	0 (0.0)	Single	
algD + pslD	6 (24)	Multiple	11 (44)
algD + pelF + pslD	5 (20)	Multiple	

Isolate symbol	Isolate code in TCP method	Phenotypic MBLs detection No. of isolates= 25	Phenotypic biofilm detection No. of isolates = 25					Molecular biofilm production			
			N n=3	S n=10	M n=8	W n=4	22 (88%) n (%)	*algD*	*pelF*	*pslD*	21 (84%) n (%)
3	1A	+	-	-	-	+	1(4.0)	+	-	-	1(4.0)
6	5A	+	-	-	-	+	1(4.0)	+	-	-	1(4.0)
7	9A	+	-	-	+	-	1(4.0)	+	-	-	1(4.0)
8	1B	+	-	+	-	-	1(4.0)	+	+	+	1(4.0)
9	5B	+	-	-	+	-	1(4.0)	+	-	-	1(4.0)
10	9B	+	+	-	-	-	1(4.0)	-	-	-	0(0.0)
11	1C	+	-	-	+	-	1(4.0)	+	-	-	1(4.0)
12	5C	+	-	-	+	-	1(4.0)	+	-	-	1(4.0)
13	9C	+	-	+	-	-	1(4.0)	+	-	+	1(4.0)
15	1D	+	-	+	-	-	1(4.0)	+	+	+	1(4.0)
16	5D	+	-	-	+	-	1(4.0)	+	-	-	1(4.0)
19	9D	+	-	+	-	-	1(4.0)	+	-	+	1(4.0)
22	1E	+	-	-	+	-	1(4.0)	+	-	-	1(4.0)
38	5E	+	-	+	-	-	1(4.0)	+	+	+	1(4.0)
39	9E	+	-	+	-	-	1(4.0)	+	+	+	1(4.0)
41	1F	+	-	+	-	-	1(4.0)	+	-	+	1(4.0)
42	4F	+	+	-	-	-	0(0.0)	-	-	-	0(0.0)
45	10F	+	-	+	-	-	1(4.0)	+	-	+	1(4.0)
46	7F	+	-	-	-	+	1(4.0)	-	-	-	0(0.0)
50	1G	+	-	+	-	-	1(4.0)	+	+	+	1(4.0)
61	4G	+	-	-	+	-	1(0.0)	+	-	+	1(4.0)
63	7G	+	-	-	-	+	0(0.0)	+	-	-	1(4.0)
80	10G	+	-	-	+	-	1(4.0)	+	-	-	1(4.0)
88	3H	+	-	+	-	-	1(4.0)	+	-	+	1(4.0)
118	7H	+	+	-	-	-	0(0.0)	-	-	-	0(0.0)

N, non; S, strong; M, medium; W, weak.

DISCUSSION:

The increase in carbapenemases and MBLs producing *P. aeruginosa* in the world raises the concern of health organizations because this increase constitutes a burden on the societies and economies of countries. Iraq is not isolated from this threat, local studies documented an increase in the phenomenon of the spread of strains with MDR and high virulence among patients, but the presence of this phenomenon among burn patients increases the difficulty of recovery and reduces the chances of survive [10, 11].

The emergence of MDR-isolates renders antibiotics intended to treat infections brought on by *P. aeruginosa* therapeutically ineffective [12-15]. The present study found 27% of the studied isolated were MBLs-producers. Other studies have also reported different prevalence of carbapenems resistance. Studies in Europe and the USA recorded a prevalence of 34.2% and 23.7% of meropenem-resistant *P. aeruginosa* strains, respectively. According to studies conducted in 36 countries (Europe, Africa, Asia and Latin America), imipenem-resistant *P. aeruginosa* was found in 47% of the strains [12]. Furthermore, a study in Poland showed that 56.2% of non-mucoid-*P. aeruginosa* strains and meropenem resistance was present in 72.2% of mucoid strains. *P. aeruginosa* strains that were resistant

to (meropenem and imipenem) were found to be present in Brazil with a prevalence of 37% and 36%, respectively [16].

The current study highlighted the possibility of finding a relationship between the MBLs-production and increased *P. aeruginosa* virulence such as biofilm production. Depending to the results of this investigation, 88% of MBLs producing isolates were also biofilm producers; therefore, there could be a real and direct relationship between MBLs and biofilm production, especially since PCR results proved beyond any doubt that there were isolates that possessed at least single biofilm encoding gene among MBLs-producers.

There are many studies that discussed the possibility of a relationship between MBLs-production and the growing characteristics of *P. aeruginosa* virulence. In order to effectively treat infections brought on by *P. aeruginosa* strains, Tuon et al [2] came to the conclusion that biofilm development among MBLs-producers is a major difficulty. The intricate structure of the *P. aeruginosa* biofilm adds to the pathogenicity of this bacterium, resulting to treatment failure and causes chronic and complex infections that are difficult to eradicate. In the same context, Breidenstein et al [17] confirmed that *P. aeruginosa* has evolved the mechanisms of its antibiotic resistance via a number of distinct pathways, including mutation-driven resistance and horizontal gene transfer (HGT) within biofilms layers. Heydari and Eftekhar [18] found that MBLs-production was substantially higher in biofilms-producers compared to non-producers, and the results were 70.3% and 31.4%, respectively, and noticed that the degree of biofilms production increases with the increase in the production of strong biofilms by the strains that produced AmpC and MBLs, as well as in the isolates that produce all classes of β-lactamases such as: "AmpC and MBLs and ESBLs", while the strains that formed one type of β-lactamases were producing weak biofilm or did not form biofilm. In another study, Chakraborty et al [19] found a significant relationship between MBLs-production in *P. aeruginosa* and the degree of biofilms formation. In a somewhat similar investigation, Perez et al [20] revealed that MBLs-producers harbored MBLs genes formed strong biofilms.

In this research, 88% of MBLs-producers had the capability to produce biofilm and 16% of them were weak biofilm formers, 32% were medium biofilms formers, and 40% were strong biofilms formers. The current investigation confirmed that biofilm production at a strong and medium level within *P. aeruginosa* isolates was higher than those isolates with weak biofilm production. Numerous investigations confirmed that the most of *P. aeruginosa* strains could form strong and medium biofilms rather than weak biofilms. Jabalameli et al [21] detected 22.9%, 26%, and 47% were produce weak, moderate and strong biofilm, respectively among 69% of *P. aeruginosa* isolates were identified from wound infections. In another research carried out by Vasiljevic et al [22] evaluated the capacity of 163 *P. aeruginosa* strains isolated from infected burn wounds to produce biofilms and found that 97.55% were biofilm-producers. Among these 163 isolates, 23.93%, 34.36% and 39.26% were produced weak, moderate and strong biofilm producer, respectively. In an investigation conducted by Banar et al [9] investigated 55/ 57 (96.5%) strains of *P. aeruginosa* were

characterize by their capacity to produce biofilms and found 30.9%, 47.3%, and 21.8% of these isolates formed strong, moderate and weak biofilm layers, respectively. Ghasemian et al [23] also reported strong, medium and weak biofilm production of 42.5%, 35% and 22.5%, respectively. In this research, it was evident that there was a statistically significant association between MBLs and biofilms formation and these findings are in line with those of Heydari and Eftekhar [18], Ghasemian et al [23], and Singhai et al [24].

Antimicrobial resistance and biofilms formation has been shown to have a synergistic impact in strains of *P. aeruginosa*, and many reports have shown that biofilm production was higher in *P. aeruginosa* strains with multiple antimicrobial resistance. The importance of the biofilm lies through its role in increasing the possibility of horizontal transfer of antimicrobial resistant encoding genes and protecting the bacterial cell from antibiotics. Bacteria with antimicrobial resistance form stronger and more resistant biofilms. Rajabi et al [25] believe that Inconsistency in biofilm diagnostic rates among *P. aeruginosa* strains and the severity of biofilm production may be linked to differences in infection origin, study sample size and geographic regions or biofilm assay methods that were often used in the investigations.

According to biofilm results in the current study, the *algD* gene found as more prevalent than other biofilm encoding genes. These findings are similar to study that conducted by Tahmasebi et al [26] that found *algD* gene in (65.4%) of *P. aeruginosa* infected burn wounds. There are other studies similar to the current study, especially in the distribution of common genes responsible for encoding biofilm production in *P. aeruginosa* strains. Rajabi et al [25] revealed that the *algD* genes were the most common, with a rate of 78.6%. Ghanem et al [27] observed a high prevalence of *algD* gene among the isolates of *P. aeruginosa* (80%), and confirmed that the *algD* gene regulates the formation of capsule by *P. aeruginosa*. It was also found that the association of biofilm formation expression in *P. aeruginosa* acts as a blocker that prevents the direct access of the antibiotic into the cells of the organism and hinders the therapeutic activity of antibiotics. It was documented that *algD* is up-regulated in biofilm producing *P. aeruginosa*.

Although the *pslA* gene was not found individually, it was found associated with other genes under study, such as *algD* and *pelF*. It is interesting that there is a relationship between these interrelated genes and the strength of biofilm formation because the isolates of this type of genes were clearly expressed in biofilm-producing isolates. Strong and medium while this pattern was not observed in isolates producing weak biofilm. The *pslA* gene presence has been considered as a good indicator of biofilm production in *P. aeruginosa* strains in several imvestigations [28, 29]. Heydari and Eftekhar [18] revealed that all isolates of *P. aeruginosa* that produces MBLs and biofilms had the *pslA* gene, indicating a strong relationship between carriage of *pslA* gene and biofilm formation.

Similar to the findings of other investigations, the current investigation revealed the clear roles of the genes (*algD*, *pslD*, and *pelF*) in production of biofilm and strength degree, where Kamali et al [30] found that the prevalence rate (87.5%) pattern of common *algD*, *pslD*,

and *pelF* genes from *P. aeruginosa* strains, which was similar to that reported by Banar et al [9]. The researchers concluded that there was a relationship between formation of biofilms and multiple antibiotic resistances by different mechanisms, such as the production of MBLs. Also found that strains producing biofilm were more resistant to multiple antibiotics. In another study, Da Costa Lima et al [31] revealed that 48.40% of biofilms-producing *P. aeruginosa* were MDR-strains. Abidi et al [32] demonstrated that the production of biofilms was substantially greater in MDR strains. Mechanisms of resistance to biofilm-producing *P. aeruginosa* to antibiotics are attributed via a number of distinct pathways, including mutation-driven resistance and HGT [17]. The importance of biofilm production by *P. aeruginosa* is because biofilm enables these pathogens to survive in the host's organs and provides them with protection from host's immune systems and antimicrobial agents. Moreover, MDR *P. aeruginosa* may arise as a result of biofilms acting as a reservoir for genes encoding antimicrobial resistance [33].

CONCLUSIONS:

According to the findings of this investigation, there was a high prevalence of biofilm-producers in MBLs-producing *P. aeruginosa* isolates and most of the biofilms-producers showed strong and intermediate ability to produce biofilm more than the isolates with weak biofilm production. The frequency of the single and associated *algD* gene was more dominant than the rest of the genes encoding biofilms production. The frequency of associated genes: *pslD*, *pelF*, and *algD* were the most dominant among the strong and intermediate biofilm producers. Thus, It can be also concluded that there was a clear positive correlation between the multiple genes encoding biofilm and the degree of biofilm production among MBLs-producers and close association between MBLs and biofilms production, which explains the prevalence of carbapenems resistance and biofilm-production in this study because biofilm provides the appropriate environment for antibiotic resistance in general and carbapenems in particular.

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