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EVALUATION OF BIOFILM FORMATION IN *PSEUDOMONAS AERUGINOSA* ISOLATED FROM CLINICAL SAMPLES AND THE PRESENCE OF BIOFILM-RELATED GENES (*pelA, pslD* AND *algD*)

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Abstract: *Pseudomonas aeruginosa* is identified as an opportunistic pathogen since it predominantly creates nosocomial infections in immune compromised people; total of 20 samples of *P. aeruginosa* obtained from a variety of locations in Baghdad hospitals isolated from: burns, wounds and sputum collected from October 2021 to the April 2022, isolates cultured by Using culture conditions, biochemical assays, and the VITEK-2 compact system. For all bacterial isolates, Biofilm production was detected using the micro titration plate technique, with the majority of strains (50%) producing moderate biofilms, 35% producing strong biofilms, and 15% producing weak biofilms. When it comes to virulence genes, In 20 isolates, three genes were identified, the *algD* gene was found in 17 isolates (85%), *pelA* was found in 17 isolates (85%), and *pslD* was found in 15 isolates (75%). The genes *algD*, *pslD*, and *pelA* were chosen as virulence factors, the genotypic *algD/pslD/pelA* – pattern, which is responsible for the phenotypic pattern of biofilm development. There were statistically significant variations in genotypic pattern predominance across biofilm forming isolates from various sources, Using Chi-square analysis, a very significant relationship between biofilm forming capability and genetic pattern (p value 0.764) was discovered.

Keywords: Biofilm formation; *Pseudomonas aeruginosa*; *pelA*; Polysaccharide; exopolysaccharides (EPS)

INTRODUCTION

Pseudomonas aeruginosa a gram negative bacteria and is the most commonly connected Pseudomonas strain with human illnesses (Streeter and Katouli 2016), Those are responsible for cystic fibrosis patients' persistent lung infections, in particular ventilator-associated pneumonia, wound infections in severe burn patients, septicemia, and catheter-associated (UTLs), multi kinds of infectious diseases have been identified linked to *P. aeruginosa,* all of which have complicated etiologies (Streeter and Katouli 2016). (1)

As a result, *Pseudomonas aeruginosa* is a widespread nosocomial pathogenic bacterium in hospitals and burn clinics, where it is a leading cause of death. (2)

Planktonic *P. aeruginosa* cells attach to surfaces and adopt a sessile lifestyle in response to environmental stimuli. Sessile cells multiply on surfaces, eventually developing into biofilms, which are encased in a self-made matrix made up of several macromolecules, including exopolysaccharides (EPS) (2).

In Gram negative bacteria biofilms, polysaccharides (a simple sugar molecule) which serve as a key role in antimicrobial resistance and in the formation of mature biofilms. (3)

Pseudomonas aeruginosa may produce a wide range of exopolysaccharides, including: ((Pel) or pellicle formation locus), (polysaccharide synthesis locus or (Psl)), and alginate.

Exopolysaccharides and extracellular DNA are significant elements that aid in the formation of huge aggregates or microcolonies, which are hallmark of biofilms. (4)

((Pel) or pellicle formation locus) include the *pelA* gene that responsible for biofilm formation. Pel serves as a major structural frame for the population, ensuring cell-to-cell connections in the *P. aeruginosa* biofilm. It also has a secondary effect of increasing antibiotic resistance to aminoglycosides, albeit only in biofilm populations. (5).

(Polysaccharide synthesis locus or (Psl)) contain the *pslD* which is a type of protein secretion involved in the exopolysaccharide export process, which makes it necessary for biofilm development. (6)

Alginate has *algD* enzyme that is responsible for converting (GDP manose) into (GDP mannuronic acid). (6)

The main gene that stimulates alginate synthesis is *algD*, which is followed by the joint action of *mucA* and *algU*. (7)

According to that, in the current study we aimed to:

investigate the frequency of the Genes carried by *P. aeruginosa*, *pelA*, *pslD* and *algD*, and how they are associated with pathogenicity above the averae of other bacterial infections in hospitals. In addition evaluation the apparent correlation of polysaccharides genes among biofilm producing *P. aeruginosa* clinical isolates.

MATERIALS AND METHODS

Ethics statement

This research was performed in accordance with theprinciples of the Declaration of Helsinki. The study was conducted according to the institutional review board (IRB) standards for research and ethics approval of the relevant institutional review boards (Department of Biology, and College of Science at the University of Çankiri, (2021). the clinical information for patients with wounds, burns, sputum was provided by Baghdad hospitals, For this type of isolates, written informed consent was obtained from hospitals and laboratory directors.

The research study was performed in accordance with the Declaration of hospitals Baghdad/Iraq guidelines.

Bacterial isolation and characterization

All specimens were cultivated on MacConkey agar (8), and incubated the plates aerobically for 24 hours at 37°C. The pure colony of bacteria was then chosen to undergo 64 biochemical tests, including oxidase, catalase, lactose, and additional, as well as confirmation of the isolates' identification using the VITEK2 compact system and device-specific diagnostic kits for *P.aeruginosa*.

Biofilm formation assay

Biofilm cultivation in 96 microplates on brain heart broth (BHI) as described by (9), And incubated at a temperature of 37°C for 24-18 hours, and after the end of the incubation period, we transfer 3-1 young and pure colonies to test tubes containing 5 mL of saline solution, to form stuck bacteria and compare growth with McFarland bacterial solutions.

180 µL of brain heart broth (BHI) were put into the wells of the plate, after that each well received 20 µL of corrected 0.5 McFarland bacterial solutions. A lid was put over the inoculation plate, and aerobically incubated under static circumstances for 24 hours at 37°C; gently the plate was cleaned three times with distilled water and set aside to dry at room temperature for 15 minutes.

The plates were shaken in order to remove all non-adherent bacteria. and was stained by 200 microliters of 0.1% crystal violet solution at ambient temperature for 15 minutes ; after any remaining stains were wiped away with clean water, the plate was left to dry for 20 minutes, the adherent cell was solubilized with 200 microliter of ethanol 95%.

A microplate reader At 630 nm, the optical density of each well was determined, for data

simplification and computation, the classification of bacterial adherence by presented in table 1 was utilized, which was based on OD values obtained for individual bacterial isolates. (10). (Table 1).

Table 1 Microtiter plate categorization of bacterial adhesion

Mean OD	Biofilm intensity
OD > 4 ODc	Strong
OD <= ODc*	Non – adherent
2ODc> OD> ODc	Weak
4ODc> OD> 2ODc	Moderate

Average OD of the negative control + (3*Standered Deviations) is equal to Cut-off value (ODc).

Molecular detection of biofilm genes

Extraction bacterial DNA

The method for extracting genomic DNA that was utilized by (11) with some modification and according to the manufacture instruction by (CBS, Scientific/USA) company: Inoculated the *P. aeruginosa* isolates into 5 mL of culture broth and incubated overnight at 37°C till the OD 600 value on a spectrophotometer reaches 0.8-1.0 and Putting the 2 mL of cultivated bacterium cell in eppendorf tubes and it was harvested through centrifugation at 13.000 rpm for 1 minute and that removed completely the supernatant.

The bacterial pellet was Re-suspend fully in 300 µL buffer MG with continue mixing the pellet and leftover supernatant by vortex or pipette to get a homogenous solution, The cells were lysed by adding combine of (20 µL Proteinase K, 5 µL RNase A Solution and 300 µL buffer MG) and it must vortex strongly to re-suspend and through incubation for 15 minutes at 65°C with taking in account inverting the tube 5–6 times throughout the incubation period to ensure thorough lysis.

Adding 250 µL Buffer MB to the lysate after complete lysis, and mix 5 to 6 times by pipetting or gently inverted.

Spin down the lid after mixing to eliminate any drips to equilibrate the genomic DNA binding to the column membrane and adding the 250 mL of 80% ethanol further in into lysate and gently invert 5 to 6 times to mix and eliminating any drips by Spin down the lid after mixing.

Filled a 2.0 mL collection tube halfway pipette with 750 µL of the 80% ethanol that will be placed in the spin column and discard the flow-through and collecting tubes after centrifuging at 13.000 rpm (1 minute) and Placed the spin column into a new 2.0 mL collection tube (additionally supplied) and Filled the spin column with 700 µL Buffer MW and Centrifuged

for 1 minute at 13.000 rpm with make sure of removing flow-through to dry the membrane and centrifuge for another minute to remove any ethanol (reuse the collection tube).and Placed the spin column into a new 1.5 mL tube (not supplied), and adding directly onto the membrane the 50 ~ 100 µL Buffer ME and Incubated for 1 min at room temperature, and finally centrifuged them at 13.000 rpm for 1 minute elution and getting a pure DNA.

Using variation of concentration (1.5 - 2 µL) of the template DNA for polymerase chain reaction (PCR).

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The algD* gene *primer

Primer	Sequence	Tm (°C)	GC (%)	Product size
algD-F	5'-CTACATCGAGACCGTCTGCC - 3'	64	72.35	593 base pair
algD-R	5'-GCATCAACGAACCGAGCATC- 3’	62	70.30	

The İngredients used in Maxime PCR PreMix kit (i- Taq) material was (DNTPs (2.5 mM), Reaction buffer (10X) (1X), i-Taq DNA Polymerase (5U/µL), Gel loading buffer(1X).
The Component used in diagnosis this gene was (Forward primer and Reverse primer (1 µL), Final volume (25 µL), Distill water (16.5 µL), DNA (1.5 µL), Taq PCR PreMix (5 µL).
The Optimum condition identification for *algD* gene by initial denaturation at 94°C for five minutes (one cycle), and 35 cycles at 94°C for 40 s, 60°C for 40 s, 72°C for 40 s, and final extension step at 72°C for 7 min (one cycle).

The *pelA* gene primer

Primer	Sequence	Tm (°C)	G C (%)	Product size
F	5'-CATACCTTCAGCCATCCGTTCTTC - 3'	72	73.88	786 base pair
R	5'-CGCATTCGCCGCACTCAG- 3’	60	71.33	

The same ingrdients of *algD* and the Optimum condition identification for *pelA* gene by initial denaturation at 95°C for five minutes (one cycle), and 35 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and final extension step at 72°C for 7 min (one cycle).

The *pslD* gene primer
The same ingrdients and optimum condition of detection *algD* gene.

Primer	Sequence	Tm (°C)	GC (%)	product size
F	5'-TGTACACCGTGCTCAACGAC - 3'	62	70.30	369 Base pair
R	5'-CTTCCGGCCCGATCTTCATC- 3’	64	72.35	

Agarose gel preparation
The agarose gel was generated via melting agarose (1.5) g in TBE buffer (100) mL that has already been prepared at 1.5% condensation, due to the research by (12), must boiling agarose before cooling at 45-50 degrees Celsius. In the pour plate, the gel was poured, which had been prepared with an agarose support plate, after the comb had been affixed to produce holes for the samples. The gel was carefully poured and allowed to cool for 30 minutes to avoid air bubbles. The solid agarose has been carefully scraped from the comb.

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Sample preparation

We do the next steps to prepare for electrophoresis due to (13) with some modifications:
Mixed 5 µL of the supposed DNA with 3 µL of the Processor loading buffer (Intron / Korea) to be electrophoresis (loading dye).
Poured the loading process into the gel pores and exposed the loading process to 70 vcm2 electric current for 1-2 hours, till tincture crossed the gel's other side.
Position the gel in pool comprises 500 mL of distilled water and 30 µL Red safe Nucleic acid staining solution and tested the gel by a UV source with a wavelength of 336 nm as shown in Figure 1.

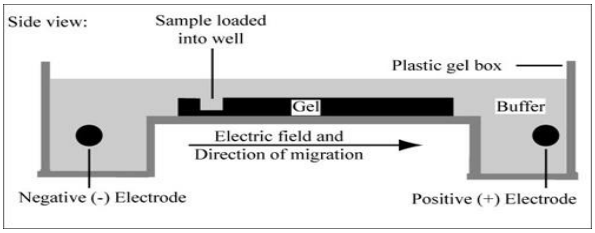

Figure 1. Working the electrophoresis system

Solution for (red safe) staining nucleic acids

At a wavelength of 537nm, Red Safe bound to DNA fluoresces. RedSafe Nucleic Acid Staining Solution's sensitivity (20.000 x) is equivalent to EtBr. This value staining (20.000x) were used by each of EtBr and Red Safe Nucleic Acid Staining Solution which in return induces a significant reduction in mutations than EtBr (putent mutagen) in the Ames test.

Specific primers for use in the procedure

the primers after they had been lyophilized, As a stock solution, to reach a final concentration of 100 pmol/µL it is dissolved in free ddH2O, and maintained as stock at -20°C to create an active primer suspension concentration of 10 pmol/µL.

Data and Static Analysis

Statistical package SPSS-22 program used for Data analysis, in which Simple percentage measurements were reflected. The significance of the difference in percentages was determined using the Chi-square test (X-) (quality data), cross table, one sample, Z-test, and the Binomial test was used to differentiate between the frequencies. When the P-value for the relevance check was equal to or less than the P-value for the main study, statistical significance was considered (0.05

Results

Bacterial Isolation and Identification

Standard identification procedures used to be able to recognize twenty (20%) *P. aeruginosa* isolates from all clinical samples, including colony characteristics on MacConky agar, the result was brownish green coloration after incubation at 37°C for an overnight period and irregular feathered edges, grape-like odor (amino acetophenone) and approximately 2 mm in diameter and lactose non fermenting (14), The findings of biochemical tests show negative reactions for methyl red, lactose and Indol, and positive reactions for catalase, gelatin hydrolysis, citrate utilization, and oxidase, the capability of VITEK®2 in recognizing *P. aeruginosa* is (99-98 %) (It indicates excellent).
Each card in the data base is based on the positivity of biological processes, with VITEK2 compact system confirmation of all isolates.

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Detection of Biofilm Characteristics in Clinical Isolates

Biofilm production was detected using the Micro titration plate technique, with findings showing that out of 20 isolates, biofilm in *P.aeruginosa* growth was divided as strong, moderate and weak. Under a set of experimental situation by using spectrophotometer technique for the qualitative biofilm formation assay, Due to OD values, the determined of cut off value (ODc) was after calculating the biofilm forming ability for each bacterial isolate and the negative control.

The three standard deviations (SD) higher than the negative control's mean OD. (15). **(FIGURE 2.** biofilm formation based on estimated cut off value of this study).

Distribution of biofilm intensity among site of infection

Isolates from wounds were divided into strong (42.85%), moderate 42.85%, and weak 14.3%, respectively, according to biofilm producers. In addition, isolates collected from burns had 28.6% strong and 71.4% moderate biofilm ability, while isolates from the sputum region had 33.33% weak, moderate and strong biofilm ability.

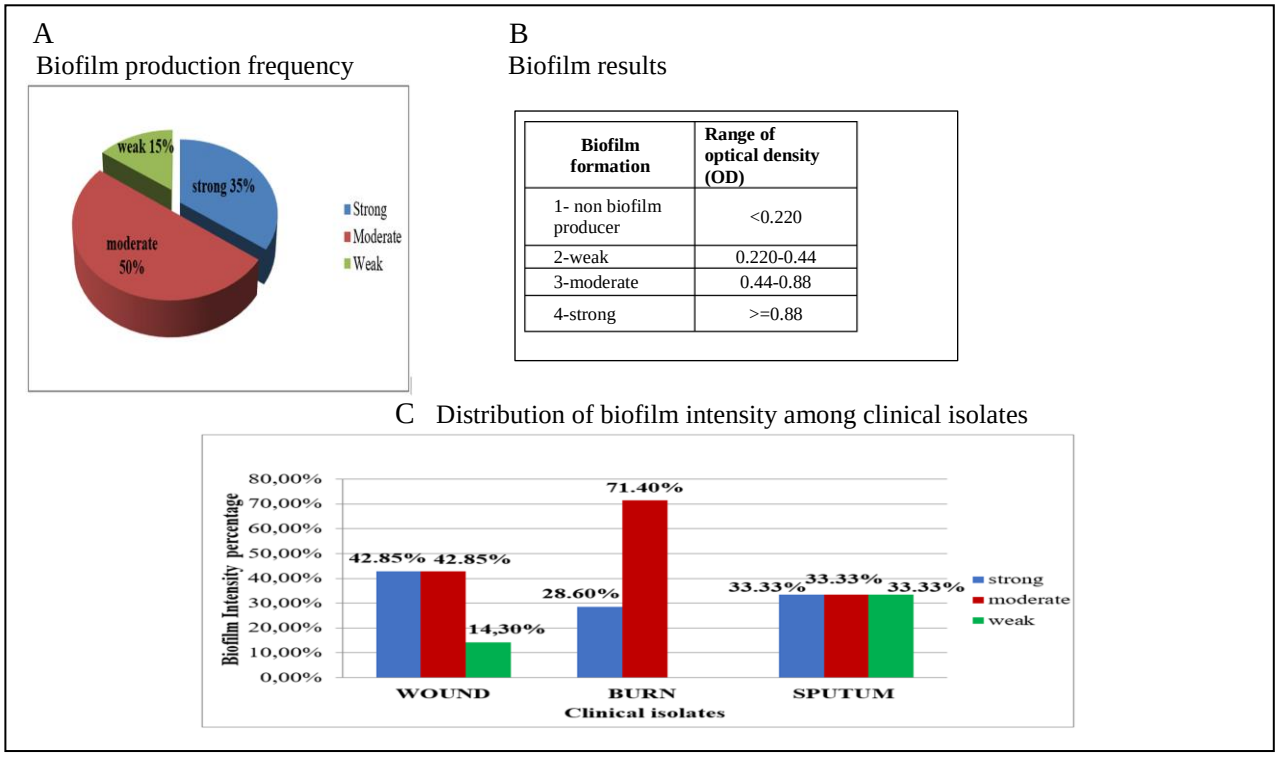

Figure 2. Biofilm results in micro titer plate according to optical density.
Cut off value = 0.220 (defined as the mean of control OD 630 plus 3 standard deviation).

Biofilm genes Detection (*algD, pelA* and *pslD*) in clinical isolates:

Instead of EtBr, we utilize RedSafe Solution (20.000x) to identify nucleic acid on agarose gels. (Item number 21141 in the catalog) (Figure 3).

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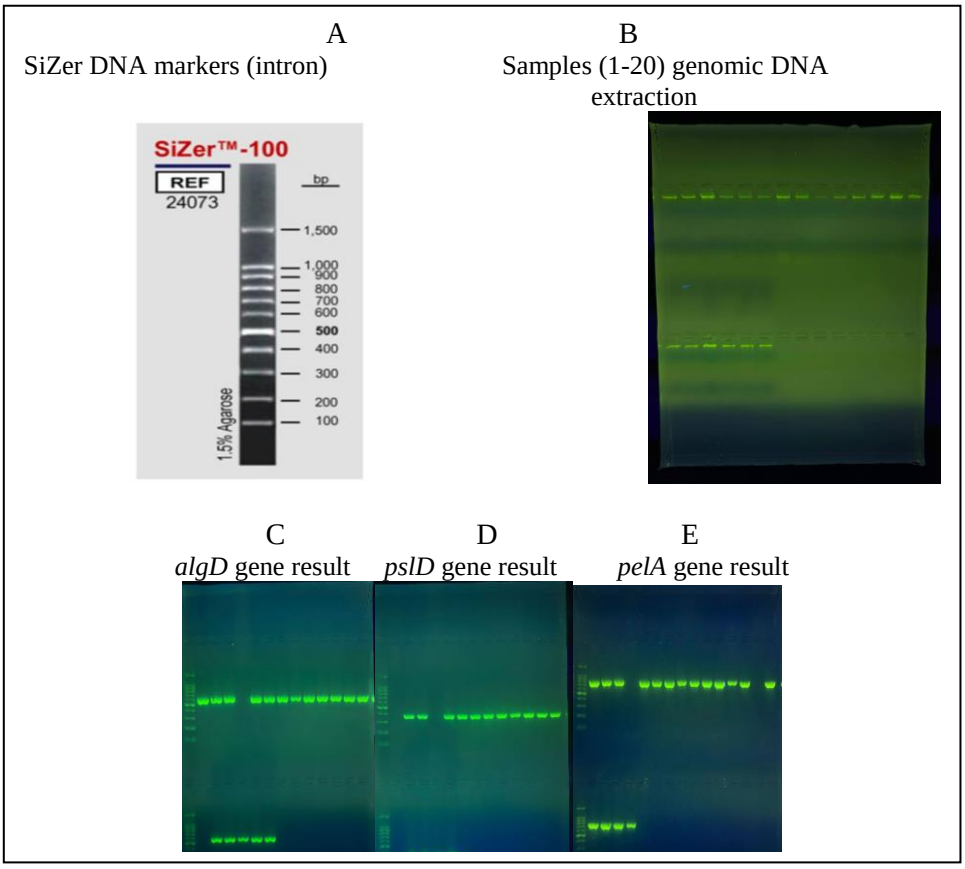

Figure 3 shows SiZer DNA markers (intron), Samples (1-20) genomic DNA extraction from bacterial samples, PCR result with a 593-bp band size for *algD* gene, The band size of the PCR result is 369 bp for *pslD* gene, Shows a PCR result with a 786-bp band size for *pelA* gene. Electrophoresis on 2% agarose at 70 volt/cm2 produced the final product. 1 hour of 1x TBE buffer N stands for DNA ladder (100).

Distribution of virulence genes among various infection sites

This study looked at the percentages of several *P. aeruginosa* virulence genes (*algD*, *pelA*, and *pslD* genes) and their connection to infection site and found positive activity was highest in sputum (100%), next pus from wounds (80%), and finally burns (75%).(figure 4).

Clinical isolates' genotypic patterns and biofilm development

Upon the appearance of these genes, 3 genotypic patterns were found, the most frequent pattern n=15 (75%) All three *algD*, *pslD*, and *pelA* genes were present in all isolates a similar time (as *algD* +/*pslD* +/*pelA* + genotypic pattern), 10% (n=2) found there are just two genes *algD* and *pelA* (as *algD* +/*pslD* -/*pelA*+), the third pattern did not harbor any genes 15% n=3 and (identified as *algD* -/*pslD* - /*pelA*). (figure 4).

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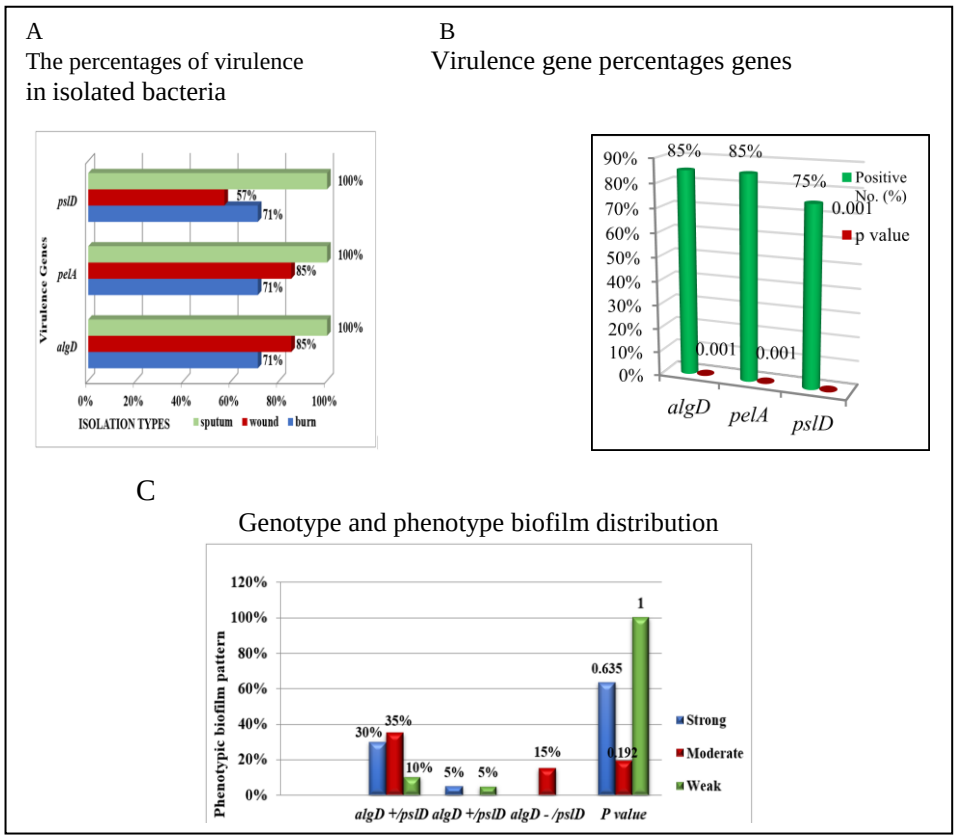

Figure 4 Virulence gene percentages in isolated infections, Genotype and phenotype biofilm distribution This statistical result are obtained by chi-square test.

Discussion

The majority of strains (50%) produced moderate biofilms, 35% produced strong biofilms, and 15% produced weak biofilms. This proportion of production of biofilms was close to the outcome of (16) which found 39% of strong biofilm and 57% for moderate biofilm producers, while the result of producing weak biofilms (15%) was close to (15) (13%) results.

The study found that 100% of the entire 20 isolates form biofilm, indicating that the bacteria have a strong capacity to form biofilms, and that this ratio is larger than the formation seen in the previous study by (17) were they recorded that *P.aeruginosa* form 94.28% biofilm production and 5.71% not production.

This clarifies the quorum sensing system function in controlling the gene expression in bacterial cell which permit the biofilm forming under un appropriate condition and otherwise it effects the pathogenicity of bacteria by controlling the biofilm formation and other virulence gene (18).

There might be differences in biofilm growth between isolates attributable to a number of variables, considering the ability of each isolate to create biofilm, the quantity and quality of quorum sensing signaling molecules, , as well as the primary number of adherent cells, produced by each isolate.

Among specimens with moderate biofilm, there is no clear pattern governing the distribution of biofilm intensity. It suggests that biofilm intensity is a specimen-dependent variable. probably the genetic material of each strain explains the observed differences (19).

In a burn infection, there are no weak biofilm producers. This might be because of the pathogen's capacity to create biofilm. (Levels of metabolic activity inside the biofilm), and their strong and

moderate biofilm intensity may be attributed to the burned percentage and depth partial thickness of burn wounds (20).

Chi-square analysis showed the same p-value (< 0.001) for burns and wound infections, while p-value (1) during respiratory infection, total p-value (0.4) for biofilm density in all samples that indicates positive results because it is less than the standard p value (0.05), and chi-square (3.65).
It shows high biofilm density in both burns and wound infections, while less biofilm density in sputum infections.

All genes had the same positive activity in burn infections 71.4%, and in respiratory infections all genes had 100% positive roles, while *algd* and *pelA* had the same positive role in wound infection 85.7%, these are close results of *algd* gene in respiratory tract infection (21) (98%) and results close to 87% of *algD* genes of burn infection in the study (22).
Except for the *pslD* gene, which had less favorable activity (57%) of outcomes in wounds, reflecting the weak role of this gene in wound infection, and Chi square analysis revealed same p value results (<0.001) in all isolate, that indicates high prevalence of (*algD, pelA* and *pslD* genes in all type of samples and that agree with the study results.

*This statistical results are obtained by chi-square test.

Biofilm Development and the Presence of Biofilm Genes

It is known that the main gene that stimulates alginate synthesis is *algD,* which is followed by the joint action of *mucA* and *algU*. (Pulcrano *et al.* 2012) and the GDP mannose dehydrogenase is the primary component of the enzyme *algD* that is responsible for converting (GDP manose) into (GDP mannuronic acid) (6).
The gene *algD* was detected in 17 isolates (85%) and other studies reported close results as (89%) by (23), (24) The *algD* gene was confirmed to be present in all of the isolates, which refer to the significant ability for alginate in biofilm formation, interfering with the antibiotic response of *P.aeruginosa* isolates (24).
Also this prevalence reflects the main function of alginate in Biofilm formation, phagocytosis and opsonization resistance, and antibiotic diffusion decrease (25).
This conclusion is consistent with the majority of findings showing alginate that is still pertinent in the subject of *P. aeruginosa* infection of CF lung mucoid strains, explaining the function of alginate production and control in chronic CF lung infection (26).
pelA is an oligogalacturone present in 17 isolates (85%) essential for Pel biosynthesis and biofilm structure, which is close to the results recorded by (87%) (23). (27)
pslD is a type of protein secretion involved in the exopolysaccharide export process, which makes it necessary for biofilm development (6). The rate of the presence of *pslD* gene found in 15 isolates is (75%), and this result relatively the same as (73.8%) due to (28).
The most prevalent virulence genes found in all 20 isolates were *algD* and *pelA* (85%), followed by *pslD* (75%), the results of *algD* and *pelA* (85%) in biofilm producing bacteria were comparable to the results found of the *algD* frequency was 87% by (29), and also in the study by (23), the frequency of *pelA* were 82.6% which is close to *pelA* frequency in our study.
Binomial test was used and the p value (< 0.001) in all biofilm genes which proved high production of biofilm with related biofilm genes.

Upon the appearance of these genes, 3 genotypic patterns were found, the most frequent pattern n=15 (75%) All three *algD*, *pslD*, and *pelA* genes were present in all isolates a similar time (as *algD* +/*pslD* +/*pelA* + genotypic pattern), 10% (n=2) found there are just two genes *algD* and *pelA* (as *algD* +/*pslD* - /*pelA*+), the third pattern did not harbor any genes 15% n=3 and (identified as *algD* - /*pslD* - /*pelA*). It revealed a significant occurrence rate of *algD, pslD* and *pelA* genes in which is close to the result

(75%) of (6) (73.8%).

The Chi-square is done by cross table and p value was performed for comparison between biofilm intensity and biofilm genes by one sample.
On the other hand, the observation of biofilm formation although no genes were detected in these isolates indicated that *P. aeruginosa* could form biofilm despite gene deficiency and could be associated with the presence of an alternative biofilm genetic factor that plays a role in metrics development, in addition may other genes have not yet been investigated, play a function in biofilm creation (27).

These results are obtained by performing the Z-test by considering the positive results as indication of success.

The results of a genotypic study (p-value 0.764) with chi-square analysis revealed an extremely important link between the production of biofilms and the genesis associated with it, that obtained high correlation between the capacity of strong biofilm and genotype pattern (p value 0.635), besides that the test showed a highly correlation between the capacity of moderate biofilm and genotype pattern (p value 0.192), and high important correlation between genotype pattern and the ability of weak biofilm forming (p value 1).
The results close to other studies by (28), (6).

Conclusion

The ability of *P. aeruginosa* to easily colonize the host and its mechanism of action through virulence factors and genes made it a critical pathogen and needs care and monitoring before it worsens.
We discovered that biofilm genes were equally present in a significant proportion of samples and predominantly in respiratory tract infection and wound infection, and were less common in burn infection in addition to biofilm formation, reflecting the active role of biofilm for such injuries and associated genes (*algD*, *pslD* and *pelA*), which revealed the strong correlation between biofilm formation and biofilm related genes, through the crucial role in increasing pathogenicity in these isolates, In respect to the results of the analysis data, it revealed a significant linked between biofilm formation and biofilm genes by presence of high rate between them in clinical samples of *P. aeruginosa*.

We noticed the low positive role of *pslD* gene in wound infection which related to the lack in function of psl in pathogenicity with this type of infection.
With respect, that representing the formation of biofilm mechanism rely on alginate as essential ESP and Pel and psl polysaccharides matrices in both respiratory, wound and burn wound infection, with some modification in prevalence.
It is revealed the obvious involved of biofilm which indicates the prevalent of its related to the pathogenicity particularly in these isolates, besides that we discovered the presence of biofilm regardless of the lack of biofilm genes in some isolates and that explain role of many factors that affect production of biofilm. And all of that highlight on the existence of alternative biofilm genetic factors and their role in matrics development and we realized that other genes may not yet investigated also play a function in biofilm creation.
Depending on phenotype detection of *algD*, *pelA*, and *pslD* outcomes, we found that most of the local isolates are mucoid and that indicates presence of (alginate) as incidence of *algD* gene in isolates, which explain that biofilm formation process depends on alginate as essential ESP apart from Pel and Psl matrices.
So local isolates was found in mucoid pattern to enhance their ability of convert into chronic mode, on the other side that helps in persistent the pathogenicity of isolates via transformation in mucoidity,

furthermore the resistance to antibiotic mechanisms to evade organism immunity and antimicrobial agents.

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Author contributions: Çankiri University conceived the study idea, supervised nd drafted the manuscript. wahj-AL DNA Laboratory staff and carried out the lab work, and did some of the lab work, and Both authors read and approved and helped in editing the manuscript the final manuscript.

Conflicts of interest disclosure: The authors declare no conflicts of interest.

Data availability: dataset was generated and analyzed duringthis study and data sharing is applicable.

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