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Molecular Investigation of the Presence of (*pslD*, *pslA*, *rhlR*) Genes in *Pseudomonas aeruginosa* isolated from Burns and Wounds in Diyala city

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Received: 6 November 2023 **Accepted: 1 February 2024**

Published: 28 April 2025

DOI: https://dx.doi.org/10.24237/ASJ.03.02.833B

Abstract

Pseudomonas aeruginosa is one of the most common Gram-negative bacteria responsible for outbreaks of infection and morbidity in hospitals. The difficulty in treating them lies in their possession of a wide range of virulence factors in addition to their ability to have natural and acquired resistance to antibiotics especially the multi-drug resistance (MDR) ones. 114 samples were collected and isolated from burns and wounds patients hospitalized in Baquba Teaching Hospital, during the period from 19/9/2022 to 12/12/2022. These isolates were diagnosed using microscope diagnosis and biochemical tests, the results showed that (12) samples (10.53%) showed no growth on the culture media, while (102) samples (89.47%) showed positive growth, as they included (32) burns samples and (70) wounds samples. Then the final diagnosis was made using the VITEK2 compact system, the results showed that (43) isolates (42.16%) were belonging to the *P. aeruginosa*, while (59) isolates (57.84%) were belonging to other bacterial species out of the total collection samples which numbered (102) isolates. A test was conducted to detect the ability to form biofilms, and the results were as follows: (Strong adherent 41%; n=7), (Moderately adherent 24%; n=4) and (Weakly adherent 35%; n=6), and they were tested for sensitivity towards 12 antibiotics, and the results were as follows: CAZ 100%; CTX 100%;

CRO 100%; AMC 96%; ER 94%; MEM 76%; TOB 76%; FEP 75%; GN 70%; CIP 47%; PB 23%. The results of the sensitivity showed that 17 isolates were MDR. Sequence analysis was used to detect the presence of (*pslD*, *pslA*, *rhlR*) genes in 17 isolates of *P. aeruginosa*, and the obtained results were as follows: the presence of ***pslD*** gene at rate of (94%; n=16), ***pslA*** gene at rate of (100%; n=17), and ***rhlR*** gene at rate of (100%; n=17).

Keywords: *Pseudomonas aeruginosa*, Virulence factors, Multi-drug resistance, VITEK2 compact system, Sequence analysis.

Introduction

Pseudomonas aeruginosa bacteria are considered one of the important bacterial species because they are widely present in various environments, including water, soil, plants and human body, they are also the main cause 10-20 % of infections in hospitals, including cases of blood poisoning and infections of wounds and burns in particular in intensive care units [1,2]. *P. aeruginosa* can produce a large number of virulence factors, including: (biofilm, mucoid exopolysaccharides, pili, lipases, leukocidin, exotoxin A, lipopolysaccharides, pigments, rhamnolipids, and proteases), these virulence factors, in turn, play role in the invasion, colonization, and spread of bacteria in the host's body, weakening its immune system, and forming a resistance barrier against antibiotics [3]. The process of forming biofilms is a complex, jointed process that includes sequential and gradual stages and includes the presence of what is called chemical communication between the inside and outside of the cells, the biofilm is the most important factor among the virulence factors [4,5]. One of the important things that should be highlight is the ability of *P. aeruginosa* to be multi drug-resistance of antibiotics, as this problem has become an international problem, as it has been shown that efflux pumps constitute the highest level of resistance, coinciding with outer membrane barriers and the formation biofilms [6,7]. The *pslD* gene plays an important role in the formation of biofilms and is therefore one of the reasons for the resistance of *P. aeruginosa* to antibiotic [8]. What is worth noting is the ability of the *pslA* gene natural charge encodes during the primary stage of biofilm formation in exopolysaccharide providing structural support [9]. Many studies have confirmed that the importance of *rhlR* gene lies in its association with virulence factors [10]. The present study aimed to detect the presence of *pslD* gene, *pslA* gene and *rhlR* gene in

isolates of *P. aeruginosa* isolated from wounds and burns, as it was detection in 17 isolates using PCR.

Material and Methods

Bacterial isolation and diagnosis

One hundred and fourteen samples were collected from sources (wounds and burns). The samples were isolated from the patients from Baquba Teaching hospital, during the period from 19/9/2022 to 12/12/2022. The isolates were cultured on MacConkey agar and Blood agar, incubated at (37ºC) for (24) hrs. Isolates were identified based on morphological traits such as shape, size, color, texture and the edges of bacterial colonies. As well as the Biochemical diagnosis through several tests such as the Catalase test, Oxidase enzyme test, Methyl red test, and Indole test. In the final confirmation of the biochemical diagnostic tests, VITEK2 compact system was used that provided by the company (Biomerieux USA), such as the Catalase test, Oxidase enzyme test, Methyl red test, and Indole test. In the final confirmation of the biochemical diagnostic tests, VITEK2 compact system was used that provided by the company (Biomerieux USA).

Antibiotic susceptibility of *P. aeruginosa*

One hundred and fourteen of *P. aeruginosa* these isolates were tested for their resistance against the following (12) antibiotics, it was tested using disks method Kirby-Bauer of Mueller- Hinton agar based on [11]. The antibiotics are: Amoxicillin (100 µg); Ceftazidime (30 µg); Cefotaxime (30 µg), Ceftriaxone (10 µg), Cephalexin (30 µg), Meropenem (10 µg), Amikacin (30 µg), Tobramycin (10 µg), Gentamicin (10 µg), Erythromycin (15 µg), Polymyxin B (300 µg), Ciprofloxacin (30 µg). The multidrug-resistance (MDR) can define as a resistance to at least one gent in three antibiotics categories groups or more.

Biofilm formation in *P. aeruginosa*

Biofilm formation portability of *P. aeruginosa* was done using the Micro Titer plate method (MTP). *P. aeruginosa* was grown along overnight in trypticase soy broth (TSB) that containing (0.25%) of glucose at (37˚C), and the free cells were removed, and the biofilm was washed, for three time with a sterile phosphate buffered saline (PBS), and fixed with (150) ml of (99%) (V/V) for Crystal violet, for (20) min at room temperature. The Crystal violet it was dissolved

using (33%) (V/V) ethanol acetone (80, 20) (V/V) for (20) min and the absorbance was measured at (595) nm, and reported values of three measurements that during three consecutive days (Figure 1).

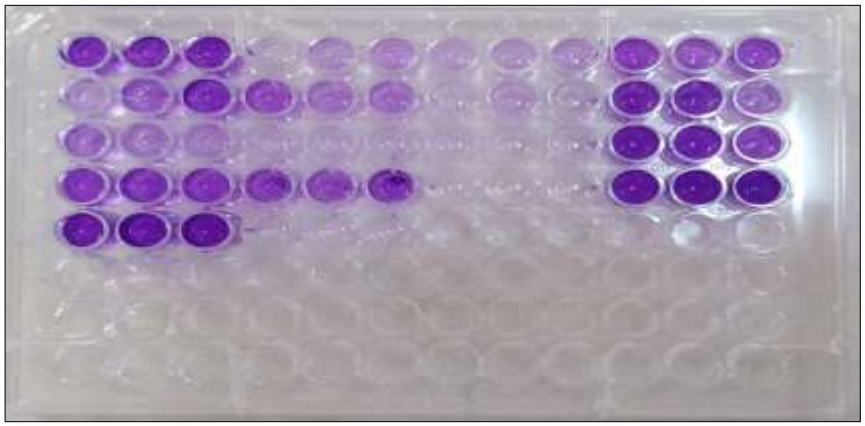

Figure 1. Micro plate showing different grades of biofilm formation

DNA Extraction of *P. aeruginosa* isolates

By the (ABIO pure^{TM} Total DNA, USA) to extracted the genomic DNA from an overnight culture. To detect the concentration of extracted DNA, was used (Quantus Fluorometer) in order to detect the goodness of a samples, for downstream applications.

Primer preparation

It was supplied by (Macrogen) Company in a lyophilized form. By a nuclease free water Lyophilized primer, it was dissolved. That to give us a final concentration of (100pmol/µl) as a stock solution, the working solution of this primer was prepared by adding (10µl) of primer stock solution (stored at freezer in-20 C) to (90µl) by a nuclease free water that to obtain the working primer solution of (10pmol/µl), (Table 1).

Table 1: Primer preparation

Primer Name	Vol. of nuclease free water (µl)	Concentration (pmol/µl)
pslD-F	300	100
pslD-F	300	100
pslA-F	300	100
pslA-R	300	100
rhlR -F	300	100
rhlR -F	300	100

Optimal PCR conditions for the detection of the *lasI* gene of *P. aeruginosa* isolates

The *pslD* gene, *pslA* gene and *rhlR* gene were detected in 17 isolates that were isolated from wounds and burns, the primer sequence is listed in (Table 2). PCR it was performed by using (Dream Tap PCR Master Mix), which contains: Taq polymerase, MgCl2, dNTPs, and the suitable buffer each PCR tube that contained (25µl) that reaction mixture composed of (12.5µl) of master mix (1µl) of each are forward and reverse primer solution. (1µl) of DNA and nuclease free water was added to complete the volume. The PCR it was performed according to the following conditions: initial denaturation at (95 °C) for (5 min) and (1cycle), then (30 cycles) of denaturation (95 °C) at (30s), annealing temperature at (52 °C) for (30s) and (30 cycles), while extensional (72°C) for (30s) and (30 cycles), followed by a final extension (72°C) for (7 min) and (1 cycle). The amplified of DNA was separated by (1%) of agarose gel electrophoresis.

Table 2: Primers annealing temperature used and sequences

Primer Name	Sequence 5`-3`	Annealing Temp. (°C)	Product size (bp)	References
pslD-F	CTCATGAAACGCACCCTCCT	52	295	[12].
pslD-R	TGCGACCGATGAACGGATAG			
pslA-F	TGGGTCTTCAAGTTCCGCTC	55	119	[12].
pslA-R	ATGCTGGTCTTGCGGATGAA			
RhlR- F	GTAGCGAGATGCAGCCGATC	57	156	[13].
RhlR -R	CCTTGGGATAGGTGCCATGG			

Results and discussion

Isolation and Identification of *P. aeruginosa*

The results showed that it was total of 114 samples isolated from wounds and burns, 12 samples (11.42%) showed a negative growth of the culture media, while 102 samples (89.47%) showed a positive growth of the culture media. *P. aeruginosa* isolates when cultured on MacConkey agar media appeared in the form of pale-colored colonies with a grape-like odor due to their inability to ferment the sugar lactose. And finally confirm diagnosis by VITEK2 compact system, where 43 isolates were obtained (42.15%) belonging to *P. aeruginosa*, they were distributed among wounds and burns, where the percentage of wounds was 37% and this percentage was close to [8], as the percentage of wounds isolates was 44%, While the

percentage of burns was 53% which is close to what it was reached by [14], who found that the burns isolates was 58%. As for the virulence factors, the ability of the isolates to produce a protease was 82% and it was identical to what was found by [15], who found that the percentage of protease production was 83%, while the ability of the isolates to produce gelatinase was 41% which is identical to what was reached by [16], who found that the percentage of gelatinase production was 45%.

Antibiotics susceptibility

P. aeruginosa isolates were detected against 12 types of antibiotics, these isolates that showed a high resistance against: Ceftazidime (100%), Cefotaxime (100%), Ceftriaxone (100%), Amoxicillin (96%), Erythromycin (94%), Meropenem (76%), Tobramycin (76%), Cefepime (75%), Gentamicin (70%), Ciprofloxacin (47) and Polymyxin (23%). These results were very close to study in Iraq [17], who found Amoxicillin (100%), Ceftazidime (100%) and Erythromycin (100%) but disagreed with [18], who found Amoxicillin (47%) and Ceftazidime (44%) and, also these results were agreed with [19], who found *P. aeruginosa* to Cefepime (71%) but disagreed with [6], that found Cefepime (38%), while Ceftriaxone and Cefotaxime agreed with [20], who found Ceftriaxone (100%) and Cefotaxime (96%) but disagreed with [21], found Ceftriaxone (30%) and [18], found Cefotaxime (40%). While [14], who found Amikacin (82%) and Tobramycin (78%) but these results disagreed with [21], who found Amikacin (27%) and Tobramycin (30%). The results also agreed with [22], who found Gentamicin (72%) but disagreed with [20], who found Gentamicin (27%), while [21], found Meropenem (77%) but [6], found Meropenem (36%), and [23], who found Polymyxin B (23%) but this result disagreed with [24], who found Polymyxin B (0%), also [25], who found the Ciprofloxacin (46%) while [24], who was found Ciprofloxacin (23%), (Figure 2).

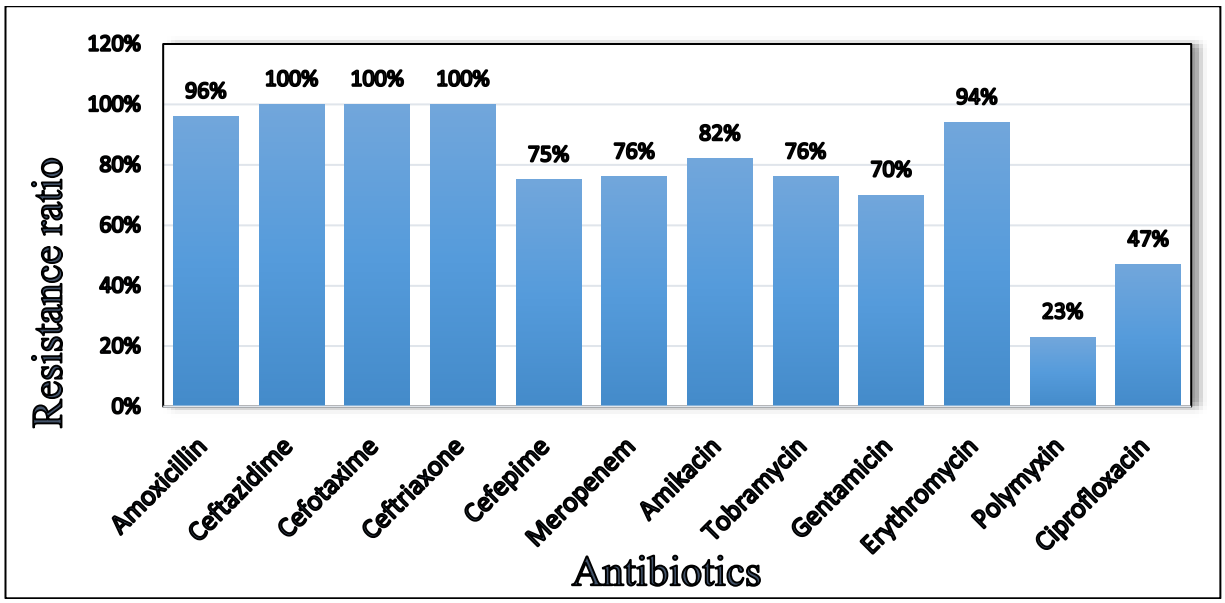

Figure 2: Antibiotics and resistance ratio of *P. aeruginosa* isolates

Returns the resistance of *P. aeruginosa* to beta-lactam antibiotics is mainly due to its production of beta lactamase enzymes that break down the beta-lactam ring, in addition to its possession of efflux pumps and its ability to change the permeability of the outer membrane., and resistance for anti- aminoglycosides due to its production of the enzymes modifiers such as: N-acetyl transferase, Phosphotransferase, and carried resistance genes on the chromosome or plasmid. While the resistance of *P. aeruginosa* to Fluoroquinolones is due to a mutation in DNA gyrase or works to inhibit DNA synthesis by stopping the action of the enzyme DNA gyrase.

Biofilm formation

The result in this study of biofilm formation were in microtiter methods after taken the mean of are three reading for 17 isolates, the results proved that all the isolates were forming biofilm and with different degrees of intensity, (Figure 3). The results Showed (41%; n=7) were strong biofilm this result was agreed with [23], who found 39% were strong biofilm, but disagreed whit [15], who found 13%. The results also indicated that (24%; n=4) were moderate biofilm this result was agreed with [15], who found 22% were moderate biofilm but disagreed with [26], who found 50% were moderate biofilm, and (35%; n=6) were weak biofilm this result was agreed with [27], that found 38% were weak biofilm, but disagreed with [22], who found 4% were weak biofilm.

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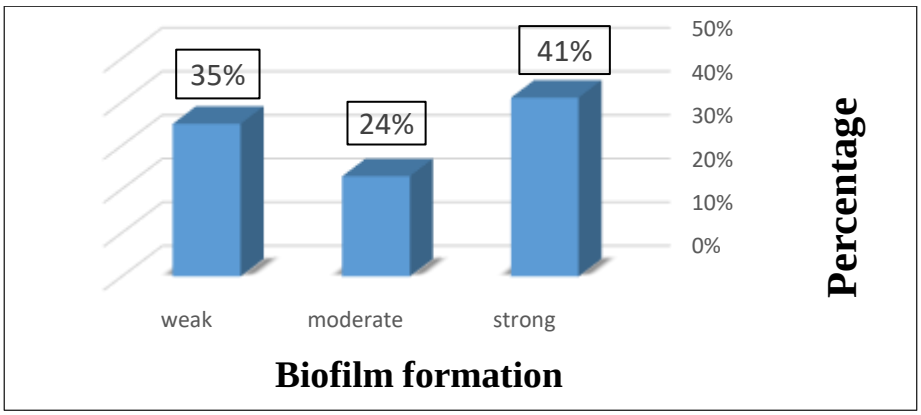

Figure 3: Biofilm formation and Percentage of *P. aeruginosa* isolates

Detection of the presence of the genes in Biofilm

1- Detection of *pslD* gene

The study show was the presence of *pslD* gene was a detected in *P. aeruginosa* in 16 isolates out of a total of 17 isolates. The detection results were showed *pslD* gene that found rate 94% for 16 isolates whereas isolate No. 4 didn't possess the gene, (Figure 4). The results of PCR of the *pslD* gene showed that it has a size of (295bp) when comparing the double bundles with the DNA Ladder size guide. These results were agreed with study conducted in China [28], who found that isolates had 96% of *pslD* gene, the results also close of study conducted in Iran [29], that found that 89% of isolates all had *pslD* gene. While these results disagreed with [30], who found 70% of isolates had *pslD* gene, also [31], who found 36% of isolates had *pslD* gene this result also disagree with the result of the current study.

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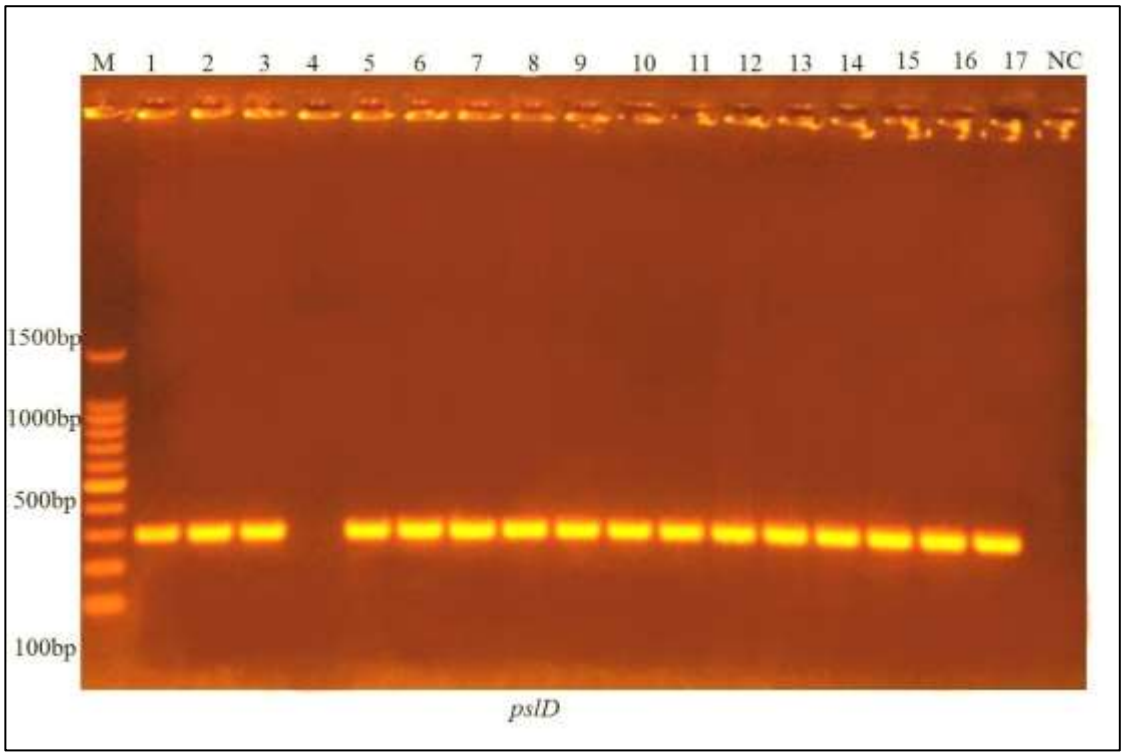

Figure 4: Results of the amplification of *pslD* gene of *Pseudomonas aeruginosa* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-17 resemble 295bp PCR products.

2- Detection of *pslA* gene

In this study was the presence of the *pslA* gene it was a detected in *P. aeruginosa* in 17 isolates. The detection results were showed that all isolates were contains of the *pslA* gene with a percentage of (100%), (Figure 5). The results were of a Polymerase Chain Reaction (PCR) of the *pslA* gene was showed that it has a size of (119bp) when comparing the double bundles with the DNA Ladder size guide. These results were agreed with study conducted in Iran [32], who found that all isolates had 100% of the *pslA* gene, the results also agreed with study conducted in Iran [33], who found that 100% of isolates all had *pslA* gene. While these results disagreed with [34], who found 83% of isolates had *pslA* gene, also [35], who found 42% of isolates had *pslA* gene this result also disagree with the result of the current study.

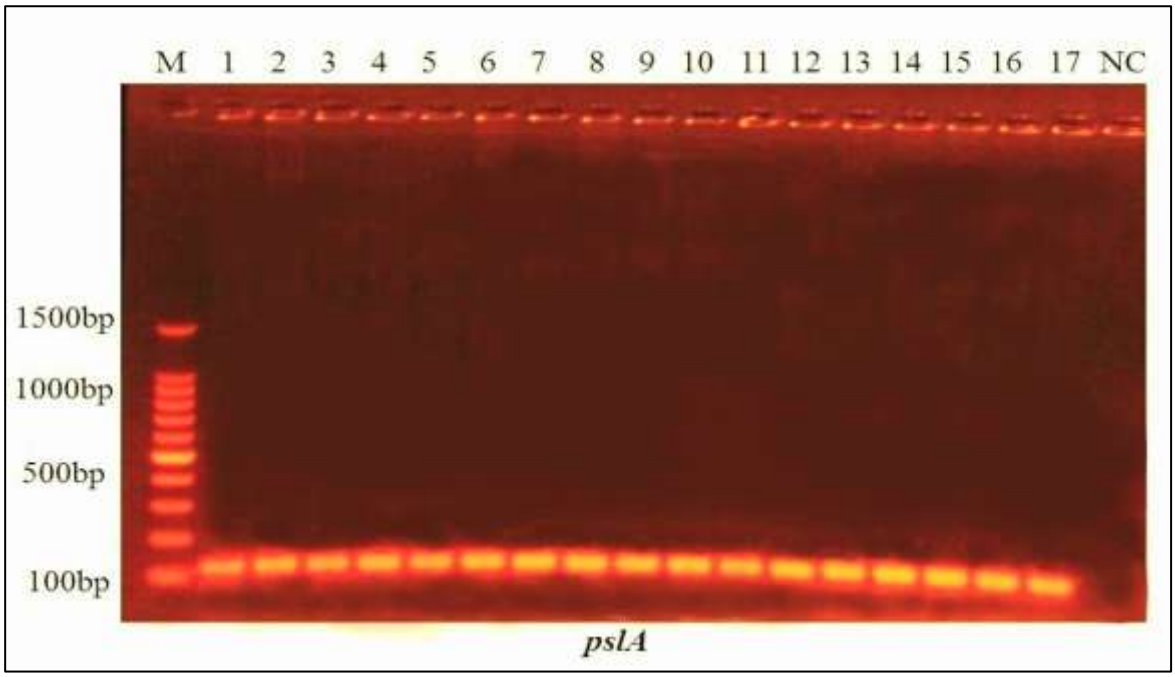

Figure 5: Results of the amplification of *pslA* gene of *Pseudomonas aeruginosa* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-17 resemble 119bp PCR products.

3- Detection of *rhlR* gene

In this study was the presence of the *rhlR* gene it was a detected in *P. aeruginosa* in (17) isolates. The detection results were showed that all isolates were contains of the *rhlR* gene with a percentage of (100%), (Figure 6). The results were of a Polymerase Chain Reaction (PCR) of the *rhlR* gene was showed that it has a size of (156bp) when comparing the double bundles with the DNA Ladder size guide. These results were agreed with study conducted in Iraq [26], who found that all isolates had 100% of the *rhlR* gene, the results also agreed with study [27], who found that 100% of isolates all had *rhlR* gene. While these results disagreed with [36], who found 81% of isolates had *rhlR* gene, also [37], who found 79% of isolates had *rhlR* gene this result also disagree with the result of the current study.

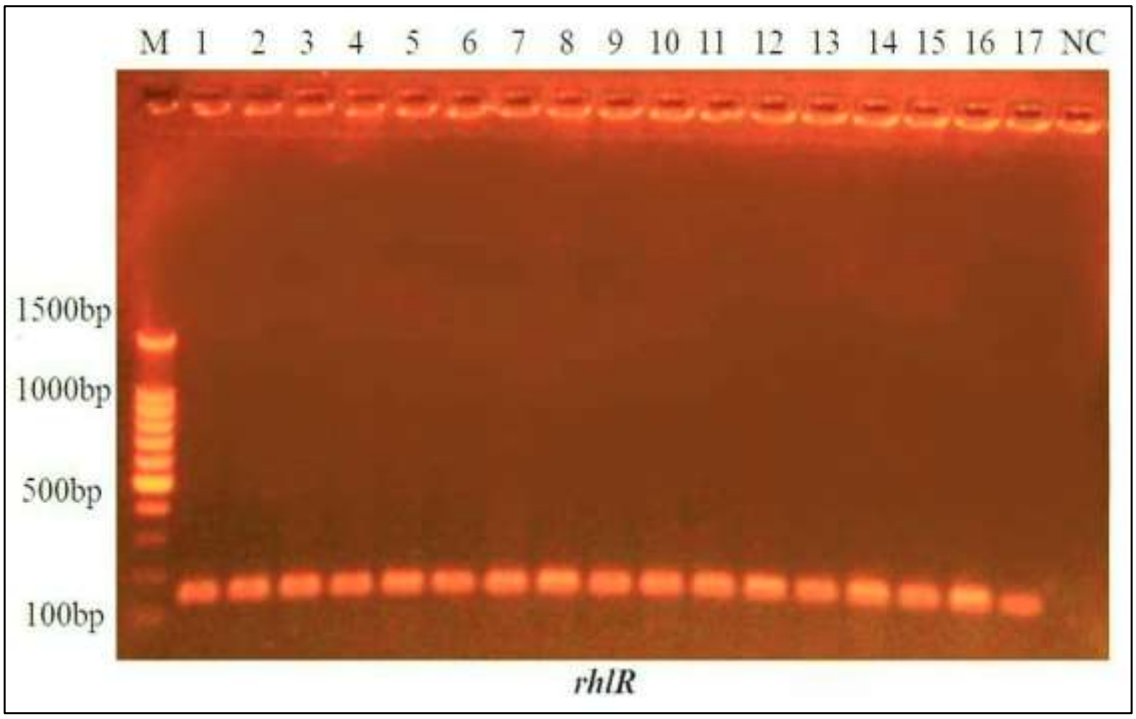

Figure 5: Results of the amplification of *rhlR* gene of *Pseudomonas aeruginosa* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-17 resemble 156bp PCR products.

Conclusion

This study was conducted on 102 isolates belonging to the *Pseudomonas aeruginosa* bacteria, which were collected from sources of wounds and burns. They were initially diagnosed using microscopic diagnosis and biochemical tests, then they were finally diagnosed using the VITEK2 compact system. A test was conducted to detect the ability to form biofilms, and the results showed that all isolates were able to form biofilms, ranging from strong, moderate, and weak. A sensitivity test was conducted towards 12 antibiotics, and it was found that all isolates showed multiple resistance to antibiotics at different rates, the least of which was anti-Polymyxin B. The presence of the pslD, pslA, and rhlR genes was investigated, and the results were as follows: the presence of the pslD gene was 94%, while the presence of the pslA and rhlR genes was 100%.

Source of funding: The researchers relied on the source of personal finance.

Conflict of interest: The researchers declare that there are no conflicts of interest in this research paper.

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