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# Prevalence of quorum sensing genes in *Pseudomonas aeruginosa* isolated from clinical specimens

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## Abstract

*Pseudomonas aeruginosa*, similar to several bacteria, use chemical signals for intercellular communication through the quorum sensing (QS) mechanism. QS enables bacterial groups to detect population density and, in reaction to variations in cell density, to synchronize their behaviors. The current work intended to identify quorum sensing virulence factor genes in *P. aeruginosa* isolated from clinical tissues. A total of 155 clinical samples were obtained from burns, wounds, urine, and ears for isolation of *P. aeruginosa*. Bacterial isolates were identified according to their biochemical reactions, then antibiotic susceptibility and Biofilm formation by isolates of *P. aeruginosa* was investigated, and the genomic DNA was extracted from each bacterial isolate for detection of quorum sensing genes (*lasR, lasI, rhlR, and rhlI*). From different clinical specimens, twenty-five *P. aeruginosa* isolates were identified. these isolates were resistant to most antibiotics, and it was found that only 10(40%) of these isolates were biofilm producers. Biofilm virulence genes *lasR, lasI, rhlR, and rhlI* were detected in all *P. aeruginosa* isolates examined. *lasR, lasI, rhlR, and rhlI* genes were common in *P. aeruginosa* isolates that have high rate of resistance to all antibiotics, and strong ability in biofilm formation.

**Keywords:** Quorum sensing, *Pseudomonas aeruginosa*, Biofilm virulence genes

## Introduction

*Pseudomonas aeruginosa* is among the most opportunistic microorganisms, bacterial infections obtained in hospitals continue to result in antibiotic resistance, which is a major healthcare issue [1]. Due to the regulation of bacterial activity and the formation of biofilms by quorum sensing (QS), *Pseudomonas* infections are frequently challenging to treat[2]. When a bacterial population reaches a certain concentration, or quorum, a bacterium known as QS is able to sense information from other cells in the population and using autoinducers, which are tiny signalling molecules, for communication purposes with them in order to regulate the synthesis of several virulence factors[3]. *Rhl* and *Las* have been recognized as *P. aeruginosa's*

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two main quorum sense systems. The autoinducer synthases lasI and *rhlI*, together with the related transcriptional regulators *lasR* and *rhlR*, comprise each system. Moreover, *P. aeruginosa* has the integrated QS (IQS) system, a newly discovered fourth system, and pseudomonas quinolone signal (PQS), a third QS system[4]. In order to orchestrate the production of various virulence factors in response to a variety of environmental stimuli at different infection sites, *P. aeruginosa*, which is an important nosocomial pathogen, utilises a complex network of hierarchical quorum-sensing (QS) systems. These QS systems include the *las*, *rhl*, and 2-alkyl-4-quinolone-related QS systems. Biofilm production is crucial to pathogenicity and antibiotic resistance[5]. This study was designed to gather information on the existence, distribution, and occurrence rate of quorum sensing in *P. aeruginosa* clinical isolates regarding their capacity to form biofilms.

## Materials and Methods

### Clinical Specimens

A total of 155 clinical samples were collected from patients ranging in age from one to sixty years old who were receiving medical care at Al-Imamain Teaching Hospital, Baghdad Teaching Hospital, Al-Karkh General Hospital, and Al-Yarmouk Teaching Hospital throughout the months of September and November in the year 2023. Otitis from the ears, burns, and wounds were among the samples that were collected. Urine from urinary tract infections was also included. After being rapidly cultured on MacConkey agar, the swap samples were next incubated at 37 degrees Celsius for a period of twenty-four hours [6].

### Identification of *Pseudomonas aeruginosa*

Identification of *P. aeruginosa* was initially performed according to their morphological and cultural characteristics, and biochemical reactions. Identification of isolates was confirmed by using automated VITEK2 system (BioMérieux, France).

### Antibiotic Susceptibility Testing

Using growth-based technologies, the VITEK 2 system is computerized microbiology software. The arrangement of the bacterial suspension followed the guidelines provided by the manufacturers. A suitable number of colonies were taken out of the overnight pure culture and placed in a 12 x 75 mm transparent polystyrene test tube with 3.0 ml of sterile saline. DensiChek turbidity meter was used to set the turbidity to 0.5 McFarland. Subsequently, the VITEK 2 compact system with GN-ID employed the same suspension. Lastly, the specimen suspension tubes and the GN-ID cassette were placed into the VITEK 2 chamber[7].

### Biofilm formation and quantification

Utilizing Luria Bertani (LB) broth medium, 96-well microtiter plates (MTP) were used to evaluate the in vitro biofilm growth utilizing the following protocols[8]: Once the identified strains of bacteria were diluted in LB broth at a ratio of 1:100, they were then seeded onto a microtiter plate with an initial turbidity of 0.05 at 600 nm. Finally, after being cultivated in the broth for the night, the microtiter plate was seeded with 200 µl of each well. The mean was calculated after each strain was examined three times in three different studies. Control wells numbered eight. The exhausted wells held sterile LB broth. The plates were then kept in an aerobic environment at 37 °C for a whole day. After the wells were incubated, their contents were decanted. Each was thoroughly rinsed three times with 200 µl of 0.9 percent sterile saline. Once the biofilm layer had adhered to each MTP well, 150 µl of 0.1% crystal violet was stained and allowed to stand

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at room temperature for a duration of 15 minutes. Following staining, excess dye was removed from the MTP by immersing it in a large container of water using a pipette[9]. The strains may be categorised as non-biofilm producers (0), weak biofilm producers (+ or 1), moderate biofilm producers (++ or 2), and strong biofilm producers (+++ or 3). This classification is based on the OD values that were calculated before. For the purpose of this computation, it is essential to keep in mind that the ODc value should not be removed from the average OD value of the strain. The following will make the interpretation of the results easier to understand: OD ≤ODc=no biofilm producer; ODc<OD≤2*ODc=weak biofilm producer; 2*ODc<OD≤4*ODc=moderate biofilm producer; 4*ODc<OD=strong biofilm producer.

**DNA extraction**

The Wizard Genomic DNA Purification Kit, manufactured by Promega in the United States, was used to extract genomic DNA from every single bacterial isolate. The efficiency of the chromosomal DNA extraction was validated by the process of electrophoresis of the DNA extracts on a 1% agarose gel that had been stained with ethidium bromide. Within the scope of this study, the NanoDrop 2000 spectrophotometer was used to assess the levels of purity and concentration.

**Amplification of biofilm genes**

The genes *lasI*, *lasR*, *rhlI*, and *rhlR* were shown to be involved in the creation of biofilms in *P. aeruginosa*. These genes were found to contribute to the formation of biofilms. Amplification by polymerase chain reaction (PCR) was performed using the primers that are specified in table (1).

**Table (1): Oligonucleotide primers used for amplification of biofilm genes**

| Gene | Sequences (5' → 3') | $T_m$ (℃) | Product size (bp) | Reference |
|---|---|---|---|---|
| *lasI* | F:GTGTTCAAGGAGCGCAAAG<br>R:GAAACGGCTGAGTTCCCAGA | 62 | 240 | [10] |
| *lasR* | F: AGATCCTGTTCGGCCTGTTG<br>R:CTGCTTTCGCGTCTGGTAGA | 62.5 | 194 | |
| *rhlI* | F:GCTACCGGCATCAGGTCTTC<br>R:GTTTGCGGATGGTCGAACTG | 63.5 | 100 | |

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| | | | | |
|---|---|---|---|---|
| *rhlR* | F:ACCAGCAGAACATCTCCAGC<br>R:CATTGCAGGATCTCGCGTTC | 64 | 157 | |

**Primer's preparation**: In order to achieve a stock solution with a final concentration of 100 pmol/µl, the lyophilised primers that were indicated in Table 2 were reconstituted in water that was free of nuclease. For the purpose of achieving a working primer concentration of 10 pmol/µl, a working primer solution was created by combining 10 µl of primer stock solution with 90 µl of nuclease-free water.

**Optimization of PCR conditions:** Amplification of biofilm genes was carried out using thermal cycler (Fisher Scientific, USA). Optimum conditions for amplification of these genes described in Table (2).

**Table (2): PCR amplification program for *P. aeruginosa* biofilm genes**

| Step | Temperature (°C) | Time | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 2 minutes | 1 |
| Denaturation | 95 | 30 seconds | 35 |
| Annealing | 57 | | |
| Extension | 72 | | |
| Final extension | 72 | 5 minutes | 1 |

## Results and Discussion

### Isolation and identification of *P. aeruginosa*

For isolation of *P. aeruginosa*, clinical samples were cultured on enrichment and differential media. From these samples, a total of 102 bacterial isolates were obtained. Different bacterial colonies were examined according their cultural and microscopic characteristics. Colonies suspected to be *P. aeruginosa* appeared as slightly curved, short to medium size, grouped as single or pairs, non-spore forming, non-motile, non-lactose fermenter, negative for Gram staining. They produce blue-green pyocyanin pigments that gave them the appearance of pale-yellow colonies. These bacterial isolates were further subjected to biochemical identification. Results showed that among the total isolates, only 25 isolates were identified as *P. aeruginosa* , as they were positive for Catalase, Oxidase, Gelatinase, and Hemolysine production tests. While they were negative for Indole and Lactose fermentation tests as indicated in table (3). There was a confirmation of the positive identification of *P. aeruginosa* isolates by the use of the Vitek 2 compact system.

**Table (3): Biochemical test results for identification of *P. aeruginosa* isolates**

| Biochemical test | Result |
|---|---|

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| | |
|---|---|
| Catalase | + |
| Oxidase | + |
| Indole | - |
| Hemolysine production | + |
| Lactose fermintation | - |
| Motility test | + |
| Gelatinase production | + |
| Grow at 42℃ | + |
| Grow at 4℃ | - |
| Gram staining | - |

**(+): Positive result; (-): Negative result**

The high isolation rate was from burn infections (50%), wound infections (24.5%), urinary tract infections (14.7%), and otitis media (10.8%). Whereas the rest 77 isolates were related to other pathogenic bacteria from different genera (*Klebsiella pneumonia*, *Proteus mirabilis*, *Staphylococcus aureus* and *Escherichia coli*).

The study focused on isolating *Pseudomonas aeruginosa* from clinical samples using enrichment and differential media, resulting in 102 bacterial isolates. Twenty-five of them were identified as *P. aeruginosa* by means of the Vitek 2 compact system, biochemical testing, microscopy, and culture.

**Antibiotic Susceptibility of *P. aeruginosa* isolates**

The Kirby-Bauer disc diffusion technique was used in order to ascertain the antibiotic susceptibility of the *P. aeruginosa* isolates. Results depicted in figure (1) demonstrated that multidrug resistance was prevailing in these clinical isolates, with 100% of the bacterial isolates exhibiting resistance to Amoxcillin-clavululanate, 96% were resistant to Amoxcillin and Cefazolin, 88% to Tetracycline, 64% to Colistin, 48% to Levofloxacin ,44% to Aztreonam , 40% to Ceftazidim and Cefepime, 36 % to Piperacillin-sulbactum, 28% to Ceftazidime-avibactam,, 24% to Tobramycin and Ciprofloxacin, 20 % to Ceftolozane-tazobactam and Meropenem-vaborbactam 16% to was resistance Doripenem, and 4% to Meropenem. Results also showed that (100%) of the *P. aeruginosa* isolates from burn, wound, UTI, and otitis infections had multi drug resistance to more than three antibiotic groups as shown in figure (1).

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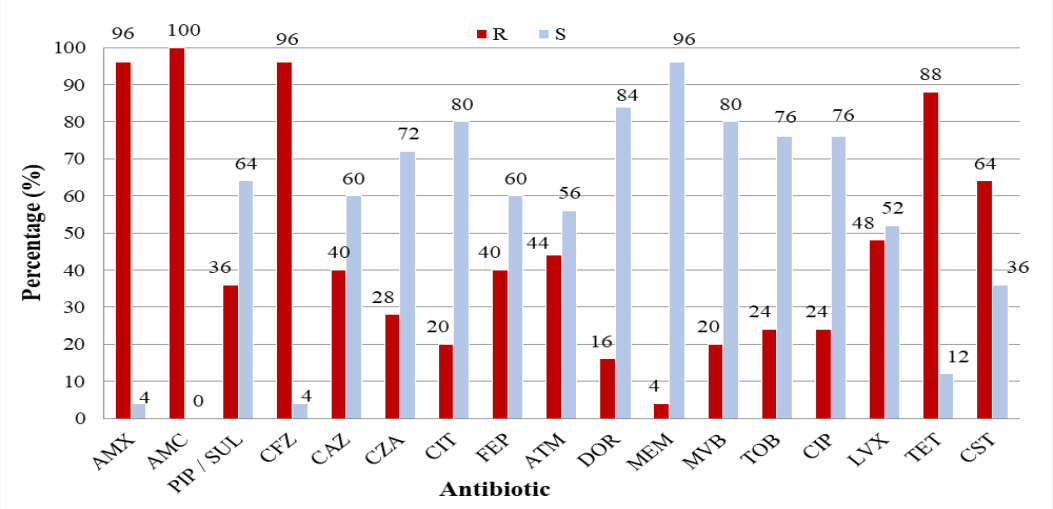


**Figure (1): Antibiogram of *P. aeruginosa* isolates and the susceptibility percentage against different antibiotics. Amx: Amoxcillin; Amc: Amoxcillin-clavululanate; Pip/sulb:Piperacillin sulbactum; CFZ: Cefazolin; CAZ:Ceftazidim; CZA:Ceftazidime-avibactam; C/T:Ceftolozane-tazobactam; FEP:Cefepime; ATM: Aztreonam; DOR: Doripenem; MEM: Meropenem; MVB:Meropenem-vaborbactam; TOB: Tobramycin; CIP: Ciprofloxacin; LVX: Levofloxacin; TET: Tetracycline; CST: Colistin.**

The antibiotic susceptibility results and resistance pattern of the current study that showed in Figure (1), were in agreement with Chika *et al.* [11], who found that 100% of *P. aeruginosa* isolates were multi-drug-resistant *P. aeruginosa*. The high rate of multidrug resistance may be caused by haphazard antibiotic use. Additionally, There are many ways that MDR *P. aeruginosa* might develop resistance, including the formation of β-lactamases, enzymes that change aminoglycosides, efflux pumps that are resistant to several drugs, and a decrease in the permeability of the outer membrane. *P. aeruginosa* strains isolated from burn patients admitted to hospitals in Tehran demonstrated a rise in the rate of multiple drug resistance due to the improper use of antibiotics[12]. Additionally, changes in bacterial enzymes and the overuse or improper administration of antibiotics by doctors and their patients may be linked to resistance.

**Ability of *P. aeruginosa* isolates in biofilm formation**

The microtiter plate method was used in order to evaluate the capacity of *P. aeruginosa* isolates to generate biofilm. Results showed as in figure (2) that all bacterial isolates (100%) were biofilm formants with variable degrees, as there is 21(84%) of the isolates were weak biofilm formats, 3(12%) were moderate biofilm formats, and only 1 isolate (4%) was strong biofilm format.

The study evaluated that all isolates were confirmed to produce biofilm, demonstrating varying strengths as showed in figure 2. Biofilms have remarkable capabilities in terms of both their physical and physiological adaptability to antimicrobial drugs. As a result of their method for improving antimicrobial

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tolerance, biofilms are able to withstand longer exposure to typical antimicrobial treatments, which allows them to keep their cell viability[13].

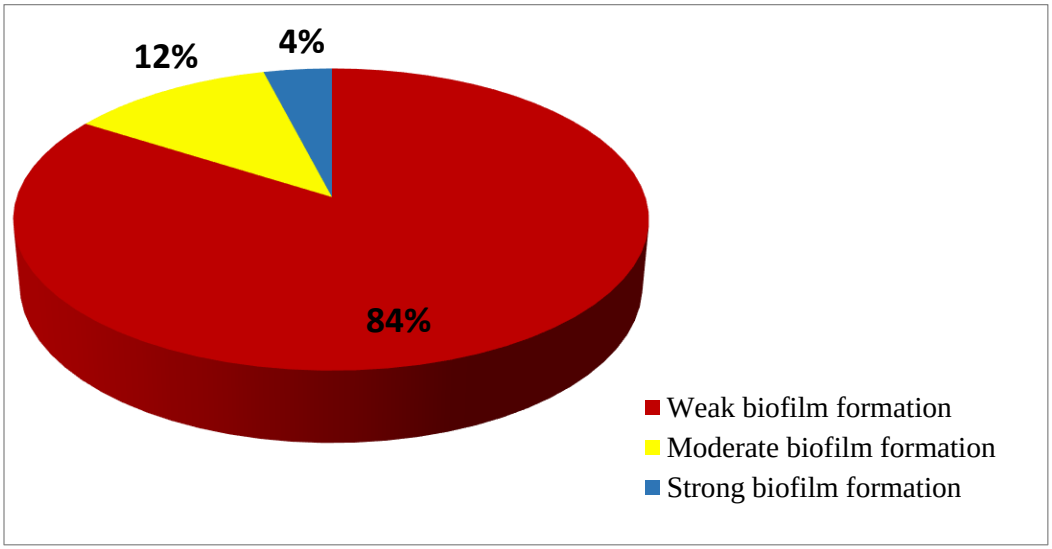


**Figure (2): Ability of *P. aeruginosa* isolates in biofilm formation DNA Extraction and Purification**

**DNA Extraction and Purification**

Twenty-five *P. aeruginosa* samples were used to extract genomic DNA using the Wizard genomic DNA purification kit (Promega, USA). When the extracted DNA was stained with EB dye, it was verified by gel electrophoresis on a 1% agarose gel and seen under UV light[20], as shown in Figure 3.

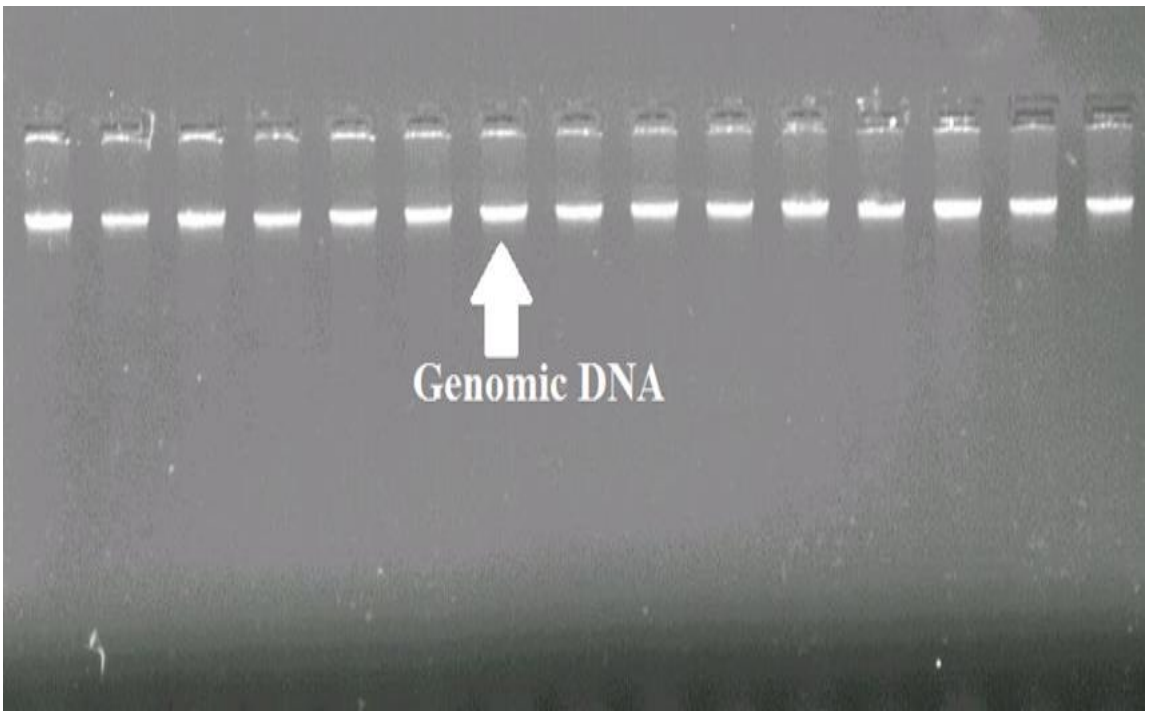

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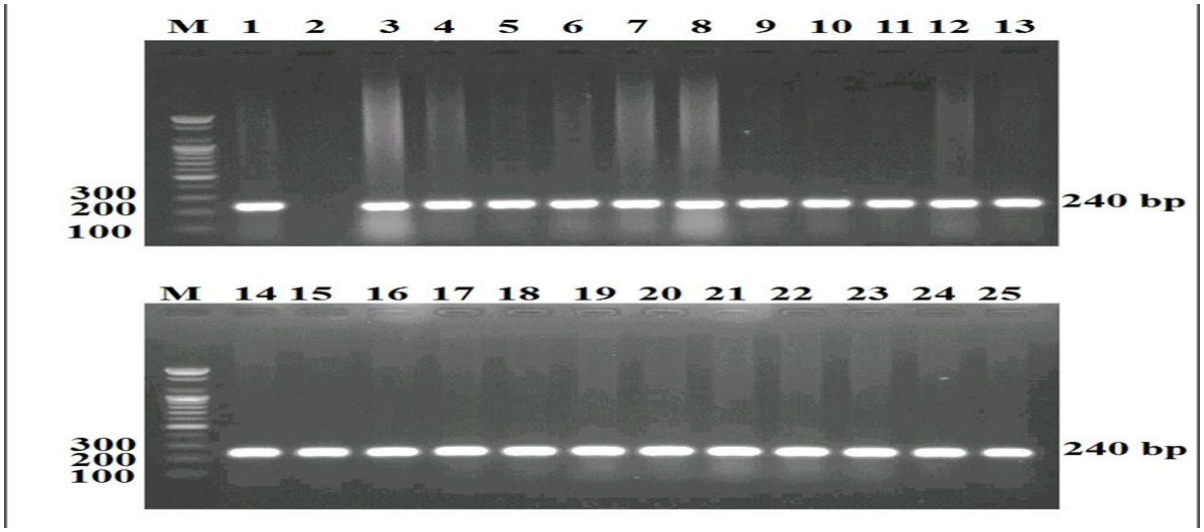
**Figure (3): Electrophoresis of genomic DNA on 1% agarose gel after extraction from *P. aeruginosa* isolates**

**Molecular Detection of Biofilm Genes**

Biofilm genes were detected in *P. aeruginosa* isolates. These genes are quorum sensing genes belong to two types, the first is auto inducers genes (*lasI* and *rhlI*), the second is transcription regulatory genes (*lasR* and *rhlR*) [14] .The virulence genes were amplified with particular primers, and the resultant products were visualised on agarose gel.

**Molecular Detection of biofilm auto inducer gene *lasI***

*LasI* was amplified using certain primers to determine its frequency among the 25 *P. aeruginosa* isolates. The amplified product was then subjected to 1.5% agarose gel analysis. Results illustrated in figure(4) showed an amplified product of 240 bp was visualized on agarose gel represents




*lasI* gene prevalent in 24(96%) isolates of *P. aeruginosa*.

**Figure (4): *lasI* gene detection after amplification of genomic DNA of *P. aeruginosa* isolates and electrophoresis on agarose gel (1.5%) for one hour. Lane (M): DNA Ladder marker; Lanes (1-25): *P. aeruginosa* isolates.**

The results of the current study regarding biofilm auto inducer gene *lasI* referred that *lasI* gene may be differently distributed in clinical isolates and was compatible with those obtained by Limaa who found that percentage *lasI* occurrence was found to be 97.5% in *P. aeruginosa* isolates. As though Sabharwal *et al*. revealed that *lasI* was detected in 75% of *P. aeruginosa* isolated from urinary tract infections [15].

**Molecular Detection of biofilm auto inducer gene *lasR***

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As shown in figure **(**5), PCR was employed to amplify the *lasR* gene in 25 *P. aeruginosa* isolates using specific primers. Results of amplification and electrophoresis on 1.5% agarose gel showed an amplified product of 194 bp was visualized on agarose gel represents *lasR* prevalent in all 25 (100%) isolates of *P. aeruginosa***.**

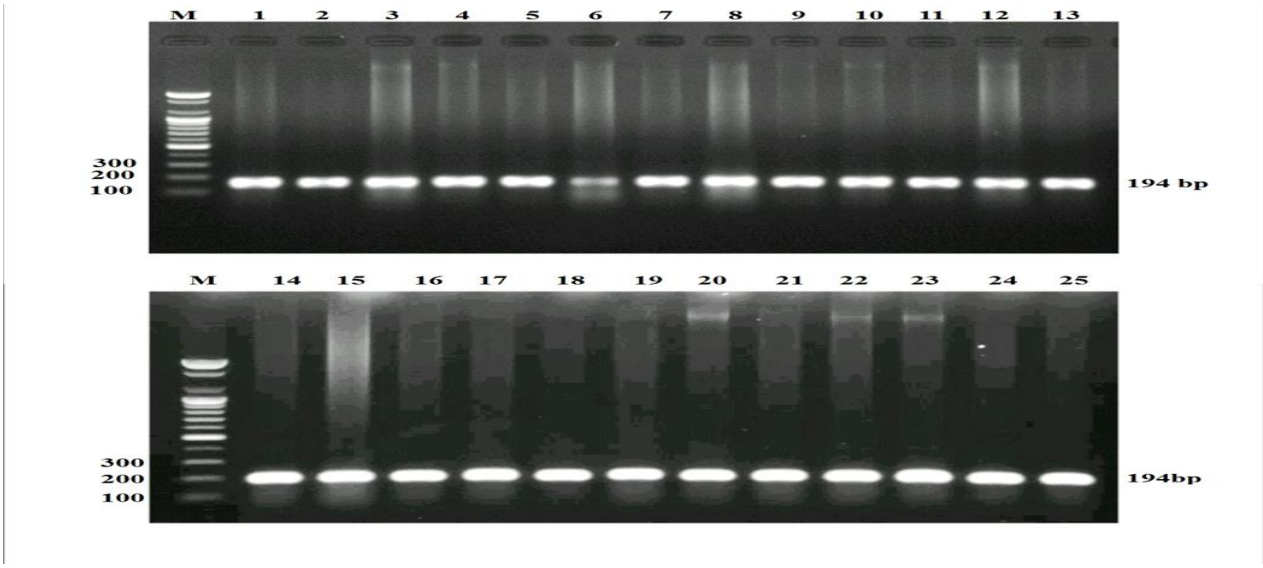


**Figure (5): *lasR* gene detection after amplification of genomic DNA of *P. aeruginosa* isolates and electrophoresis on agarose gel (1.5%) for one hour. Lane (M): DNA Ladder marker; Lanes (1-25): *P. aeruginosa* isolates.**

The *lasR* gene encodes the transcription factor that activates many target genes, primarily associated with quorum sensing in *P. aeruginosa* and findings are similar to those obtained by Lima[10] who found, *P. aeruginosa* isolates with the *lasR* were all 100% present. In contrast, Hemmati's [16] findings shown that the identification of QS genes was lower than that of the present research (78.3% for the *lasR* gene).

**Molecular Detection of biofilm auto inducer gene *RhlI***

The present investigation employed polymerase chain reaction to identify the *rhlR* gene, which encodes rhamnolipid, by amplification with particular primers to ascertain gene frequency among *P. aeruginosa* isolates. Results illustrated in figure (6) showed that *RhlI* gene was also present in all 25 (100%) bacterial isolates.

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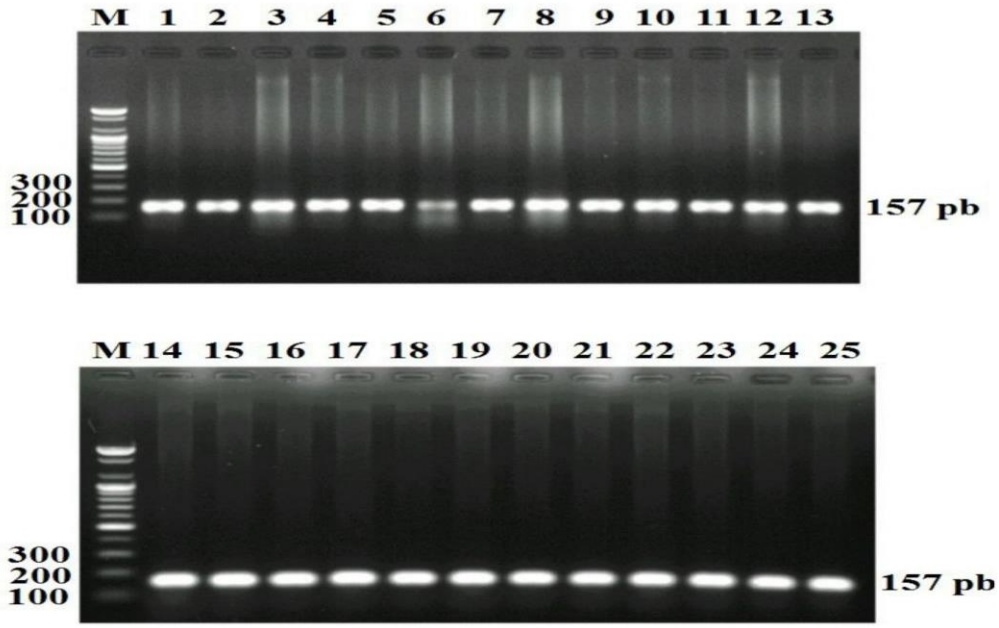
**Figure (6): *RhlI* gene detection after amplification of genomic DNA of *P. aeruginosa* isolates and**




**electrophoresis on agarose gel (1.5%) for one hour. Lane (M): DNA Ladder marker; Lanes (1-25): *P. aeruginosa* isolates.**

According to the findings of the present investigation on the biofilm auto inducer gene *RhlI*, in local research carried out by Al-Kilabi [17], 31 isolates of *P. aeruginosa* were tested for the presence of the *RhlI* gene; the results showed that 25 isolates (or 80.6%) had a positive gene frequency. Nevertheless, when the *RhlI* gene was detected molecularly in *P. aeruginosa* isolates, 56 of the isolates (93.33%) tested positive for this virulence gene [18].

**Molecular Detection of biofilm auto inducer gene (*RhlR*)**

*RhlR* gene was amplified using specific primers to determine its frequency in MDR and high pathogenicity *P. aeruginosa* isolates. Figure (7) illustrates the amplification and electrophoresis results on a 1% agarose gel, which showed that the *RhlR* gene was present in only 24(96%) isolates of *P. aeruginosa*. the genomic DNA of isolate.

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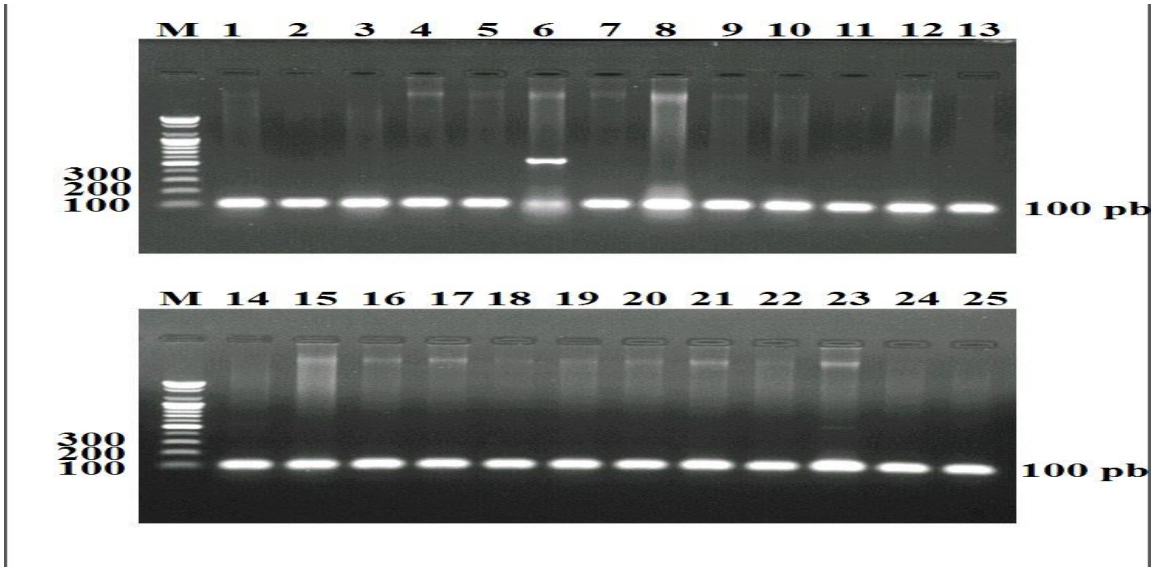


**Figure (7): *RhlR* gene detection after amplification of genomic DNA of *P. aeruginosa* isolates and electrophoresis on agarose gel (1.5%) for one hour. Lane (M): DNA Ladder marker; Lanes (1-25): *P. aeruginosa* isolates.**

The findings of biofilm auto inducer gene *RhlR* are similar to those obtained by Lima[17] that 100% of *P. aeruginosa* carrying *RhlI* gene. In another study conducted in Egypt, it was found *RhlI* gene was prevalent in 45(90%) of *P. aeruginosa* isolates[19].

## Conclusions

*lasR, lasI, rhlR, and rhlI* genes were common in *P. aeruginosa* isolates that have high rate of resistance to all antibiotics, and strong ability in biofilm formation.

## Acknowledgments

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## Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.

- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.

- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

## Author's Contribution Statement

[First Author]: participated in the collection of clinical specimens, and did experiments.

[Second Author]: Design of the study, and conceived the manuscript.

Both authors reviewed the manuscript.

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