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PHENOTYPE AND GENOTYPE DETECTION OF QUORUM SENSING IN *PESUDOMONAS AERUGINOSA* ISOLATED FROM VARIOUS CLINICAL SOURCES

Suha Hameed Mahmood and Ali J. Saleem

Department of Biology, College of Education for Pure Science, University of Diyala, Iraq.
e-mail : suhahameed147@gmail.com, alijaffar3@yahoo.com

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ABSTRACT : A total of 380 clinical samples of wound and burn infections, urinary tract infections, blood, otitis media, tonsils and vaginitis were collected from inpatient and outpatient patients at Baquba Teaching Hospital and Consultation Clinic in Diyala Governorate, during the period from 27/09/2020 to 10/1/2021. Detection using traditional methods, biochemical tests and molecular detection using the *rpsL* gene showed that 58 (15.4%) of the isolates were Pseudomonas aeruginosa bacteria. All isolates gave positive results for both oxidase and catalase assays. The DNA of 32 *P.aeruginosa* isolates was extracted by an extraction kit and the concentration of the DNA samples was measured by a nanodrop and the concentration ranged from ((31-38 ng/µl)) Polymerase chain reaction technology was used to detect quorum sensing genes (*lasI*, *lasR*, *lhII* and *lhIR*) for DNA extracted from 32 isolates of *P. aeruginosa*. The results showed that all of the 32 selected isolates were positive for (*rhII* and *rhIR*) genes by (100%) and positive for (*lasI* and *lasR*) genes by(96.7%), while one isolate gave a negative result for (*lasI* and *lasR*) genes. DNA sequencing analysis of genes (*lasI*, *lasR*, *rhII*, *rhIR*) and the results showed the presence of several mutations represented by transition mutations and substitution mutations that led to changes in amino acids.

Key words : Urinary tract, *Pseudomonas aeruginosa*, phenotype and genotype detection.

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INTRODUCTION

Pseudomonas aeruginosa is a major cause of nosocomial infections, and is the second most common pathogen isolated from patients among the Gram-negative pathogens reported by the National Nosocomial Infections Surveillance (NNIS) (Azam and Khan, 2019). These bacteria can cause a wide range of acute and chronic infectious diseases. The main sites of infection are the respiratory tract and the urinary tract, Burns, Digestive System, Ear Infections, and Wounds. *P. aeruginosa* can reach the bloodstream through these sites, which leads to a systemic infection called bacteremia (Saleem, 2012; Migiyama *et al*, 2016) and the symptoms of this infection are general inflammation and sepsis, especially in burn patients, where the skin of the host is destroyed and patients with compromised immune systems, including patients with HIV or cancer patients, who are immunosuppressed (Rostamzadeh *et al*, 2016). *P. aeruginosa* is the second most common cause of nosocomial pneumonia in hospitals, the third most common cause of urinary tract infection in hospitals, and the seventh most common cause of bacteremia (Fazeli and Momtaz, 2014; Ayat and Ali, 2021). Quorum Sensing System (QS) is defined as a chemical communication process that occurs between cells to coordinate and regulate gene expression and cell activities within bacterial communities through the production and release of molecules They are chemical signals that are diffusible and have low molecular weights called autoinducers (AI), and the chemical composition of these molecules varies according to the types of bacteria. Three types of these molecules have been discovered, namely, acyl-homoserine lactone (AHL) and these molecules are common in Gram-negative bacteria and molecules of short peptide catalysts, Oligopeptide-AIP. These are common in Gram-positive bacteria and Autoinducer-2 (AI-2) molecules are present in both Gram-negative and Gram-positive bacteria (Rai *et al*, 2015). When the concentration of these particles Outside the cell to the critical extent, sensitization is done by bacteria depending on the number

of bacteria, where the higher the number of bacteria, the stronger the communication between cells to produce these molecules in order to coordinate the work of the entire bacterial community until it behaves as a multicellular organism in the regulation of many cooperative behaviors associated with the pathogenesis and virulence of bacteria as the production of virulence determinants important for bacterial colonization, infection and biofilm formation Requirements for secondary metabolism and biodegradation of contaminants as well as antibiotic resistance (Yong *et al*, 2015; Papenfort and Bassler, 2016; Moradali *et al*, 2017). One of the quorum sensing systems that was discovered more than 25 years ago is the LuxI/LuxR system in the marine luminous bacterium fischeri Vibrio. These bacteria belong to the Gram-negative bacteria and these bacteria live in the organs (Mangwani *et al*, 2012). Illumination For many marine animals and these bacteria lights up when they are found in a large number of luminous organs and this system depends on AHL molecules for intercellular communication (Nealson *et al*, 1970). Subsequently, several quorum sensing systems were discovered and the role of AHL molecules in the regulation of genes for these systems was studied in several Gram-negative bacteria, including *Pseudomonas aeruginosa* (Mangwani *et al*, 2012).

MATERIALS AND METHODS

Sample collection

In this study was collected 380 samples under specialized medical supervision from various medical conditions and different ages of both sexes for the period between 27-9-2020 until 10-1-2021 and it included: 88samples of wound infection, 35samples of chronic otitis media, 93samples of urine, 28 samples of burns, 47samples of blood, and 49 samples of throat and 40 samples of vagina from Baquba Teaching Hospital, which were implanted in the media of the MacConkey agar, blood agar, pseudomonas agar and in the medium of Cetrimide agar for the purpose of isolation and initial diagnosis of bacteria.

Diagnosis of bacterial isolates

Phenotypic test

All isolates were identified through their growth on blood agar, MacConkey agar, pseudomonas agar, and in cetrimide agar to diagnose the culture characteristics in terms of colony shape, size, color, odor and ability to produce pigments (Levinson, 2016). Also diagnosed by the shape of colonies when stained with a gram stain, and tests for oxidase and catalase (Procop *et al*, 2017) and IMVC test (Indole test, Citrate Utilization test, Methyl – Red test, Voges-Proskauer test) (McFadden, 2000).

Detection of the ability of bacteria to produce Acyl-homoserine lactone (AHL) by colorimetric method

The AHL molecular signal was detected according to what was mentioned in Baldiris *et al* (2016) as follows:

1. The isolates were cultured on MacConkey agar medium and incubated in the incubator at 37°C for 24 hours. (4-2) 2-of the bacterial colonies were transferred and cultured in a nutrient broth and then incubated for 24 hours at a temperature of 37 °C.

2. After incubation, 1.5 ml of the bacterial culture was transferred to clean, sterile Ependroff tubes and centrifuged at a speed of (10000) cycles for (15) minutes. Then transfer the esophagus to a new small tube and discard the tube containing the sediment, then discard the new small tube containing the sediment in a centrifuge at a speed of 10,000 cycles for 15 minutes as well.

3. Then the sediment was transferred to a small tube and the precipitate was discarded. 0.5 ml of ethyl acetate was added and placed in the incubator for 1-2 hours in the incubator at a temperature of (40-42°C).

4. Then the squid was transferred to a polystyrene plate (containing 96 holes) at an amount of (40) microliters for each isolate and 2 replicates were made from each isolate. The nutrient broth without bacterial culture was also placed as a negative control.

5. 50 microliters of (1:1) NaoH solution: hydroxylamine was added to each hole.

6. (50) micro liters of (1:1) solution (10% iron chloride in 4M hydrogen chloride: 95% ethanol alcohol) was added.

7. I read the optical absorbance of all the pits with a microplate reader device at a wavelength of (630) nm, and the optical absorbance of the implanted pits was compared with the control pits and expressed as (+) the presence of AHL (-) the absence of AHL according to the following:

ODc = 0.98

ODc < ODi (+) Presence of AHL

ODc > ODi (-) No AHL.

Molecular identification

The diagnosis of *P. eruginosa* was confirmed genetically and by using the technique of polymerase chain reaction (PCR) using the *rps L* gene after DNA was extracted from *P. aeruginosa* by using the laboratory kit (Genomic DNA extraction kit), equipped by the American company ABIO pure. Extraction according to the company's instructions proven in the method of work and electrocuted in a 1.5% agarose gel.

Table 1 : The primers sequences used in the study and the volume of products after propagation.

Gene		Sequence of forward and reverse Primer(5'- 3')	Product bp	Refrence	Origin
rpsL	**F**	GCAAGCGCATGGTCGACAAGA	201	Xavier *et al*. (2010)	Alpha DNA Co.(Canada)
	R	CGCTGTGCTCTTGCAGGTTGTGA			
rhlR	**F**	CAA TGA GGA ATG ACG GAG GC	730	Senturk *et al*. (2012)	Alpha DNA Co.(Canada)
	R	GCT TCA GAT GAG GCC CAG C			
rhlI	**F**	CTT GGT CAT GAT CGA ATT GCT C	625	Senturk *et al*. (2012)	Alpha DNA Co.(Canada)
	R	ACG GCT GAC GAC CTC ACA C			
lasR	**F**	ATG GCC TTG GTT GAC GGTT	725	Senturk *et al*. (2012)	Alpha DNA Co.(Canada)
	R	GCA AGA TCA GAG AGT AAT AAG ACC CA			
lasI	**F**	ATG ATC GTA CAA ATT GGT CGG C	605	Senturk *et al*. (2012)	Alpha DNA Co.(Canada)
	R	GTC ATG AAA CCG CCA GTA G			

Table 2 : Programming of the Polymerase Chain Reaction (PCR).

Steps	Cycle	rps L
Initial Denaturation	1	5min. at 95^{0}C
Denaturation	30	30sec. at 95^{0}C
Annealing	30	30 sec. at 57^{0}C
Extension	30	30sec. at 72^{0}C
Final extension	1	7min. at 72^{0}C
Hold	1	10min. at 10^{0}C

needed to prepare the PCR mixture. A unipplex PCR was performed with a volume of 25 µl prepared by adding a microliter to each of the front and back initiator at the concentration of Pmol/µl, 12.5 µl of GoTaq®Green master Mix (2X) Promega, 3 µl of DNA template and 7.5 of deionized water. Then the device was programmed to provide The optimal conditions for performing the replication steps for the genes used in the study are as

Table 3 : Contents of multiplex PCR reaction mixture for QS genes.

No.	Contents of reaction mixture	Final concentration Pmol/µl	Volume of reaction mixture for a single tube (µl)
1	Green master mix	2x	12.5 µl
2	Each forward primer (Two.*rhlR,rhlI*). (Two.*lasR,lasI*)	10	1µl for each genefinal volum 2 µl
3	Each reverse primer (Two.*rhlR,rhlI*). (Two.*lasR,lasI*)	10	1µl for each genefinal volum 2 µl
4	DNA template		3 µl
5	Nuclease free water		7 µl
6	MgCl		0.5 µl
	Total volume		25 µl

The contents of tube mixed well by using vortex then placed in a PCR.

Primers preparation

The primers used in this study were prepared according to the company's instructions and provided by the company (Alpha DNA), whose sequences and the volume of their outputs are shown in Table 1.

Amplification of the diagnostic gene for *P. aeruginosa*

The *rpsL* Primer was used to diagnose *Pseudomonas aeruginosa*. Prepare the reaction mixture as instructed by the manufacturer Bioneer by adding the required volume and concentration of each of the components shown in Table 2. The electrophoresis of the five genes of *P.aeruginosa* was carried out by means of an aerosol gel at a concentration of 1.5% containing 1µl of ethidium bromide, with a voltage of 100 volts, for one hour.

PCR amplification FOR QS genes

Multiplex PCR assay used to detect QS genes (*rhlR, rhlI*) and (*lasI, lasR*) carried out in a total volume of 25µL, containing 5µL of template DNA, 1µL of each of the primers. then the volume was completed with 25µl of nucleases free water (Table 3).

Table 4 : PCR machine program (Thermal Cycler) for QS genes.

Step	Program	
1	One cycle for (5) minutes at a temperature of (94) °C to initial denaturation DNA template. 35 cycles included:	
2		
	A	(30) seconds at a temperature of (94)°C to denaturation DNA template.
	B	(30) seconds at a temperature of(50) °C primer annealing with DNA template.
	C	(30) seconds at a temperature of(72)°C primer extension with DNA template.
3	One cycle for (10) minutes at a temperature of (72)°C to final extension of replicated DNA strand.	

Thermal cycling conditions

The reaction performed in a PCR thermal cycler apparatus,and according to the manufacture's guide the following program was adopted: PCR consisted of a preheating at 94ºC after this initial denaturation step, the mixture was subjected to35 amplification cycles (Table 4).

RESULTS AND DISCUSSION

Isolation and identification of *Pseudomonas aeruginosa*

58 isolates of *P.aeurginosa* were obtained from a total of (380) samples with an infection rate of 15.4%, collected from different isolation sources and of different ages, including both sexes, collected from patients with infection of wounds, burns and Otitis media and urinary tract, blood and throat and vagina, for the period between 27/9/2020 until 10/1/2021 in Baquba Teaching Hospital and Consulting Clinic in Diyala Governorate. MacConkey agar and blood ager were used to grow samples. Then they were grown on selective pseudomonas isolation agar and selective cetrimide agar for the purpose of purification, and it was confirmed by biochemical tests. Isolates were diagnosed based on the phenotypic characteristics of the developing colonies, as they appeared on the MacConkey agar pale colorless due to their inability to ferment the lactose sugar present in the cultivated medium and they have a smell similar to the aroma of fermented grapes, while their colonies appeared in a dark color and most of them were surrounded by a transparent halo in the blood agar, indicating their ability for hemolysis. All isolates showed a bright green color when grown on the medium of Pseudomonas agar. This medium was considered to be selective for Pseudomonas. The results of the microscopy showed that the isolated bacterial cells of the bacilli were motile single or double-orderly negative Gram stain. The results of the biochemical tests showed positive results for the oxidase test and the catalase test in all isolates due to the ability of the bacteria to produce the enzymes oxidase and catalase. In the IMVIC assay, all isolates gave a positive result in the Citrate Utilization test as the sole carbon source, while they gave negative results with the Voges-Proskauer (VP) and MR methyl red assay and the Indole production assay.

Molecular diagnostics using the *rpsL* gene

The results of the detection of the *rps L* gene in the isolates included in the study, and they were all carrying the gene (100%), as the results of the polymerase chain reaction of the *rps L* gene showed that it has a size of 201 base pairs when comparing the replicated bundles with the volume index DNA Ladder with known beams with molecular sizes and supplied by Bioneer \ Korea, it was noticed that the packet sizes are similar to the expected size when compared with the results of Friyah and Rasheed (2019) as in Fig. 1 A, B.

Prevalence and distribution of in clinical specimens

The 58 isolates were classified according to the sites of infection after they were diagnosed according to what we mentioned. It is noted that the largest percentage of *P. aeruginosa* isolates was among the burn samples (9) isolates (32.14%) of the the total number of isolates, followed by the percentage of isolates of middle ear samples, which amounted to (9) isolates (25.7%), while the percentage of isolates in tonsil samples was (9) isolates (18.3%) of the total. As for the wound samples, the percentage was (14) isolates (15.9%) of the total isolates. As for urine isolates (11) isolates were (11.8%) and blood isolates were (4) isolates (8.5%), while vaginal isolates were (2) isolates (5%). The percentage of *P. aeruginosa* bacteria isolated from burns in this study is in agreement with the results of the researcher's study Alwasity (2018), where the percentage obtained from burns was 33.9%. As for the percentage of isolating *P. aeruginosa* bacteria from otitis media, it was in agreement with Abdul-wahid (2014), where the percentage obtained was 25%, while it did disagree with the study of Al-Wasity (2018) where the percentage of isolates they obtained was 34.8% The percentage of isolating *P. aeruginosa*

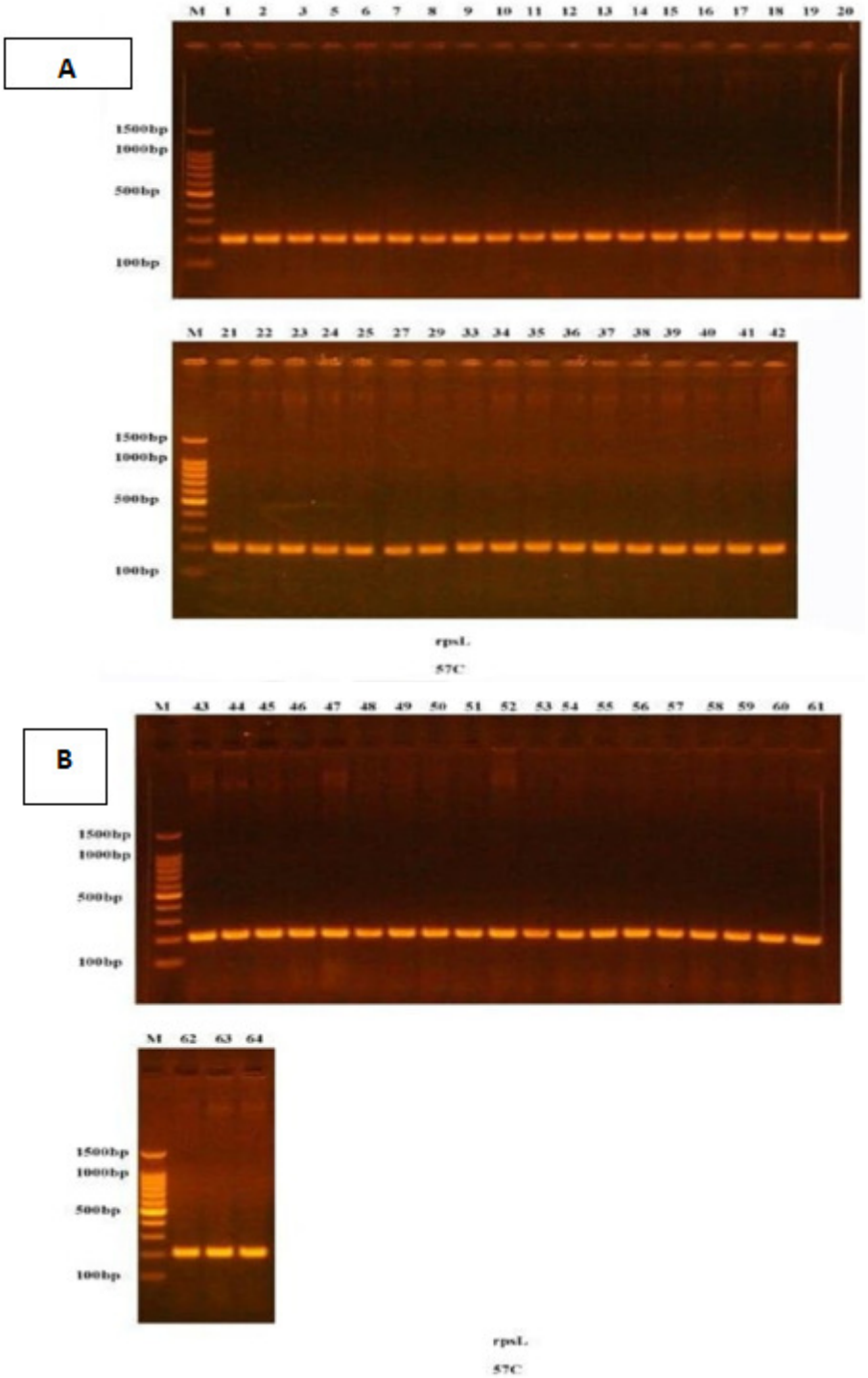

Fig. 1 : A, B- Electrophoresis transfer of PCR *of P. aeruginosa* isolates using *rps L* gene initiator (201 base pairs) were applied to an agaros gel at a concentration of 1.5% and a potential difference of 100 volts/for 60 minutes.

bacteria from samples Tonsillitis is in agreement with the percentage obtained by AL-HaiK *et al* (2016), where the percentage of isolates they obtained was 20% of the tonsillitis samples, and this result contradicted what was reached by researcher Al-Jubouri (2021), where she obtained an isolation rate of 4%. As for the wounds, they were in agreement with Al-Jubouri (2021), where they obtained an isolation rate of 14.49%. This may be due to the fact that these bacteria are considered secondary opportunistic pathogens as they take advantage of a general or local imbalance in one of the body's mechanical or immune defenses or both (Brooks *et al*, 2007; Goering *et al*, 2008). As for the urine rate, it was in agreement with Mohammed (2014), where their percentage was 12.24%, while it dis agree whith results of the researcher Najeeb (2020), as the percentage of isolation of this

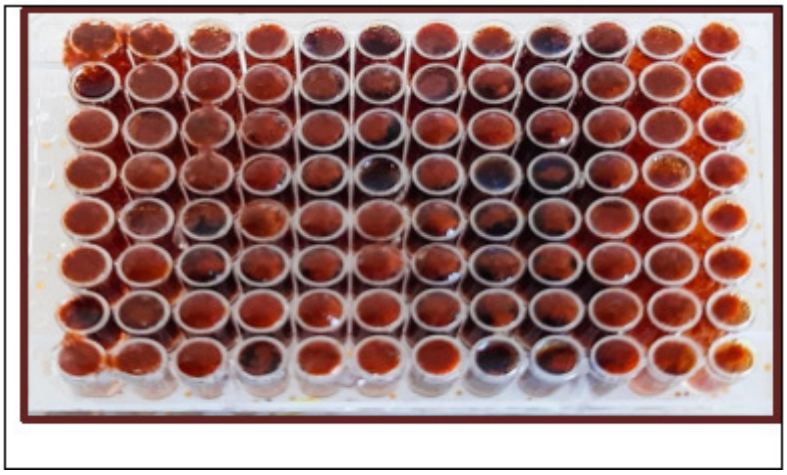

Fig. 2 : Detection of the AHL molecular signal by *P. aeruginosa.*

Table 5 : shows the percentage of AHL-producing and non-producing isolates.

AHL production	Source of isolates	The number	Percentage
+	Blood, urine, burns, wounds, otitis media, vagina, tonsils	57	98.27%
-	Blood	1	1.72%

Table 6 : Number and percentage of QS genes possessed by *P. aeruginosa.*

Genes	Wound	Burns	Urine	Blood	Otitis media	Tonsilis	Vaginal	Percent%
lasI *lasR*	+	+	+	+	+	+	+	100%
rhII *rhIR*	+	+	+	+	1-	+	+	96.8%

bacteria from urine was 4.55%. *Pseudomonas aeruginosa* is a common pathogen of urinary tract infections, especially after surgical intervention. The presence of these bacteria in the urinary tract is indicated by limited factors such as urinary catheters in urological patients, especially since most of these patients are elderly or suffer from immunosuppression for any reason, as these bacteria are characterized by their ability to colonize the surfaces of urinary catheters and form Biofilms and thus when catheterization is performed, these bacteria are transmitted to the patient's urinary system, causing urinary tract infections and resistance to many antibiotics through their presence within the (thick biofilm) (Cole *et al*, 2014).

As for the rate of isolation from blood, 8.5%, this percentage is in agreement with a study conducted by Ganjo (2017), where the percentage of isolation obtained from blood samples was 10.9%, and the percentage of isolation of bacteria from vaginitis was 5%, and this result was dis agree whith results of Abbas (2018), where the percentage of isolation was 100%.

The difference in *P. aeruginosa* isolate rates and species in local and international studies may be due to several reasons, including sample size and different seasons of sample collection, isolation times and source of isolation, and the difference in the geographical location of the sample and the number of samples and other influencing factors, as well as the most important factor is antibiotics and their misuse, attention to hygiene and the type of sterilizers and disinfectants used in hospitals.

Phenotypic and genetic detection of Quorum Sensing:4-3

Phenotypic detection of the AHL molecular signal :1-4-3

The results showed that 57 clinical isolates (98.27%) were AHL producers, where their values were greater than 0.98 and they gave a dark brown color, as shown in Fig. 2 and Table 4. One isolate was non-AHL-producing, which is isolate No. 3, and the source of this isolate was blood, as it gave a reading of 0.961 and this value is less than 0.98. The results of this study are similar to that of Sallman *et al* (2018) where the percentage obtained was 100%. The ability of the isolates under study to produce AHL is evidence of their ability to form biofilms and their ability to resist antibodies and antibiotics.

Genetic Detection of quorum sensing genes

The four QS quorum sensing genes (*lasI*, *lasR*, *rhII*

Table 7 : Sequence analysis of the DNA of the QS genes.

lasI						
Sample	**Location**	**natural sequence**	**Variation**	**Variation of amino acid**	**Type of mutation**	**Refernce Sequence ID**
3	8815877	CGA	CTA	Arginine> Leucine	Substitution	LR130528.1
22	1555387	CGA	CTA	Arginine> Leucine	Substitution	CP034908.2
35	2631451	CGA	CTA	Arginine> Leucine	Substitution	CP051770.1
47	1555387	CGA	CTA	Arginine> Leucine	Substitution	CP034908.2
lasR						
Sample	**Location**	**natural sequence**	**Variation**	**Variation of amino acid**	**Type of mutation**	**Refernce Sequence ID**
22	3002238	GGC	GGT	Threonine>Alanine	Transition	CP050323.1
rhIR						
Sample	**Location**	**natural sequence**	**Variation**	**Variation of amino acid**	**Type of mutation**	**Refernce Sequence ID**
22	3365872	GAG	AAG	Glutamate>Lysine	Transition	CP0537061
35	1670673	GAG	AAG	Glutamate>Lysine	Transition	CP053321
47	1572575	AAG	GAG	Lysine> Glutamate	Transition	LR1343091

and *rhIR*) were detected by multiplex PCR technique for the selected 32 isolates as shown in Fig.5 A, B, C. The results showed that all of the selected isolates were positive for genes (*rhII* and *rhIR*) with a percentage of (100%) and positive for genes (*lasI* and *lasR*) with a percentage of 96.7%, where isolate No. 63 gave a negative result for (*lasI* and *lasR*) genes, and the QS system for this isolate was considered deficient, while the rest of the isolates had a complete quorum sensing system as shown in Table 6.

There are studies that gave different results for these genes, where their genetic detection showed that some isolates of Pseudomonas aeruginosa were deficient in the QS quorum sensing system, including a study conducted in Iraq by the researcher AL-Shamary (2018), where it obtained percentages of 89.66%, 79.3% and 93.1% and 96.6% of these percentages were positive for *lasR*, *rhIR*, *lasI* and *rhII* genes, respectively and there is a study conducted by Al Kubaisi (2018) on *P. aeruginosa* bacteria. It showed that 82.53% of the clinical isolates carried the *lasR* - *lasI* - *rhIR* - *rhII* gene, while 15.87% were carrying the *lasI* - *rhII* - *rhIR* genes, and 1.58% were carrying the *lasR* - *lasI* - *rhIR* genes. Another study conducted in Iraq by Kadhim and Ali (2014) showed that 65%, 43.3%, 5% and 78.4% of *Pseudomonas aeruginosa* isolates were positive for lasR - rhII - rhIR genes, respectively. Senturk *et al* (2012) concluded that 77.7%, 88.8%, 66.6% and 77.7% of the isolates were positive for *rhIR* - *rhII* - *lasR* - *lasI* genes, respectively.

The selected isolates, which were 32, were all positive for the virulence factors that were conducted in the current study, represented by the possession of capsule, the possession of the biofilm, the production of protease enzyme and hemolysin, All of them were AHL-producing, except for isolate No. 3, which isolated from the blood. This isolate was not AHL-producing While it is productive for all virulence factors under study and contains the four quorum sensing genes, in such a case the researchers reasoned that the four genes of this isolate might contain disabling mutations (Karatuna and Yagei, 2010; Senturk et al, 2012). This isolate is considered non-productive for one or more virulence factors, but its quorum sensing system is complete.

DNA sequencing experiments

The DNA sequences of *lasI*, *lasR*, *rhII* and *rhIR* genes were analyzed between the results of isolates No. 3, 22, 35 and 47 isolated from blood, urine, wounds and burns that had genetic mutations in some QS genes, as shown in the Table 7.

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