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Molecular study of quorum sensing and biofilm formation genes in pseudomonas aeruginosa isolated from UTIs patients

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***Abstract*---**Background: Urinary tract infection (UTI) is a common health problem in both community and nosocomial settings, affecting both men and women equally. Pseudomonas aeruginosa is an opportunistic human pathogen causing devastating acute and chronic infections in individuals with compromised immune systems. Biofilm is an architecture built mostly by autogenic extracellular polymeric substances which function as a scaffold to encase the bacteria together on surfaces, and to protect them from environmental stresses, impedes phagocytosis and thereby conferring the capacity for colonization and long-term persistence. So, the aim of this study to screen of some important quorum sensing and biofilm genes among pseudomonas aeruginosa isolated from UTIs patients. Methods: These study was conducted in Al-Qadisiyah province, Iraq at five major hospitals (AL-Diwanyia Teaching Hospital, Feminine and children teaching hospital, Afak General Hospital, AL-Hamzah General Hospital and AL-Shamiya General Hospital) during the period from (November, 2020 to June, 2021). A total of 800 urine samples were collected from male and females referring to five major hospitals. The age of the patients ranged from (1 to 80) years-old. Results: Sixty isolates were showed positive and identified as P. aeruginosa by using selective media, biochemical test system and VITEK-2 compact system. Genetically, in the present study, a total DNA was extracted from all clinical P. aeruginosa isolates. The current study revealed that all isolates showed difference in contain the ninth genes (lasR, rhlI, pelA, pslA, lecA, ndvB, tssc1, vfr and QscR), which was related with biofilm formation and Quorum sensing (QS) phenomenon. Conclusion: The study conclude the quorum sensing system play a important role in pathogenicity and biofilm formation of P. aeruginosa,

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and it's an essential for bacteria to increase growth and resistant of antibiotics.

***Keywords*---**UTIs, P. aeruginosa, quorum sensing, biofilm.

Introduction

Urinary tract infections (UTIs) are defined as the presence of bacteria in urine along with symptoms of infection. There are three basic categories of UTI are cystitis, pyelonephritis and asymptomatic bacteriuria (1). This infection is the most commonly experienced by humans after respiratory and gastrointestinal infections and predominant cause of both hospital acquired (nosocomial) infections and community acquired for patients admitted to the hospitals (2). *Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium that causes nosocomial infections, as well as fatal infections in immunocompromised individuals, such as patients with cancer, post-surgery, severe burns or infected by human immunodeficiency virus (HIV) (3). It normally lives in moist environments, and uses a wide range of organic compounds for growth, thus giving it an exceptional ability to colonize ecological niches where nutrients are limited, from water and soil to plant and animal tissues (4).

P. aeruginosa is a well-known biofilm former, which makes it an excellent model to study biofilm formation (5). A resilient biofilm is a critical weapon for *P. aeruginosa* to compete, survive and dominate in the cystic fibrosis lung polymicrobial environment (6). *P. aeruginosa* also effectively colonizes a variety of surfaces including medical materials (urinary catheters, implants, contact lenses, etc.) (7). Common antimicrobial agents like antibiotics frequently exhibit limited efficacy due to adaptability and high intrinsic antibiotic resistance of *P. aeruginosa*, thus increasing mortality (8). Additionally, treatment of these infections is also hindered by the *P. aeruginosa* ability to form biofilms which protect them from surrounding environmental stresses, impedes phagocytosis and thereby confers capacity for colonization and long-term persistence (9). Such ability is promoted by effective cell-to-cell communications within the microbial communities of *P. aeruginosa* known as quorum sensing. As a result, highly structured biofilms can be formed which is often identified in patients with chronic infections, such as chronic lung infection, chronic wound infection and chronic rhinosinusitis (10). QS involves the production, secretion and accumulation of signaling molecules called autoinducers (AI) whose specificity and concentration are sensed by transcriptional regulators (11), resulting in the expressions of specific sets of genes on a population-wide scale. In addition to biofilm development, QS has been linked to the regulation of other physiological processes, including virulence-factor production, stress tolerance, metabolic adjustment and host-microbe interactions (12).

There are four distinct pathways in the QS circuits of *P. aeruginosa*, namely Las, Rhl, PQS and IQS that intracellularly produces their cognate AI molecules, i.e., *N*-3-oxo-dodecanoyl-L-homoserine lactone (3O-C12-HSL), *N*-butyryl-L-homoserine lactone (C4-HSL), 2-heptyl-3-hydroxy-4-quinolone (PQS) and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS), respectively. These QS circuits are organized in a

hierarchy with the Las system at the top of the cascade (13). Both Las and Rhl systems are triggered by an increased cell density at the preliminary exponential growth phase, whereas PQS and IQS systems are activated at late exponential growth phase especially under iron limitation and phosphate starvation conditions, respectively (14). The aim of the present study is molecular detection of most important quorum sensing and biofilm genes among *pseudomonas aeruginosa* isolates.

Materials and Methods

Study Design and Sample Collection

These study was conducted in Al-Qadisiyah province, Iraq at five major hospitals (AL-Diwanyia Teaching Hospital, Feminine and children teaching hospital, Afak General Hospital, AL-Hamzah General Hospital and AL-Shamiya General Hospital) during the period from (November, 2020 to June, 2021). For each patient, medical records were included name, gender, age, hospitalization, address, and antibiotic receiving. A total of 800 urine samples were collected from male and females referring to five major hospitals. The age of the patients ranged from (1 to 80) years-old.

Inclusion and Exclusion Criteria

Inclusion criteria included male and females gender, positive microbiological evidence of UTI (bacterial growth of higher than 10^5 CFU/mL), and willingness to be recruited in the study, all the *P. aeruginosa* isolates included in present study were obtained from patients specimens has UTIs without any additional substances. The study did not included any specimens with incomplete information and patients who have been on antibiotics for at least three days, and forbidden biological materials or genetically modified organisms.

Ethical Approval

All subjects involved in this work were informed and the agreement required for doing the experiments and publication of this work was obtained from each one prior the collection of samples. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee at (College of Medicine University of Al-Qadisiyah).

Identification of *P. aeruginosa* Isolates:

Depending on its morphological properties (colony form, size, color, borders, and texture), a single colony from each primary positive culture on blood, MacConkey and nutrient agar and classify it and examine it by light microscope after being stained with Gram's stain. Biochemical tests were performed on each isolate after inspection to complete the final identification according to (15) and it used the VITEK-2 compact system for *P. aeruginosa* final identification.

Antibiotic Susceptibility Testing

Antibiogram testing was performed with the automated VITEK-2 compact system based on the MIC technique determination by using AST-N222. Any isolates of bacteria that were resistance to at least one antibiotic in three or more classes called Multi Drug Resistance (MDR), isolates were resistant to at least one antibiotic in all but one or two classes of antibiotics called Extensive Drug Resistance (XDR), the isolates exhibited resistance to all classes of antimicrobial agents called Pan Drug Resistance (PDR) (16).

DNA extraction

This method was made according to the genomic Anatolia DNA purification kit supplemented by the manufacturing company.

Detection of some of *P. aeruginosa* quorum sensing and biofilm genes:

DNA (extract from bacterial cells) was used as a template in specific PCRs for the detection of some of *P. aeruginosa* quorum sensing genes. DNA was purified from bacterial cells by using the Anatolia DNA Purification Kit. The primers used for the amplification of a fragment gene and thermal cycling conditions were listed in Table (2-1), (2-2).

Table 2-1
The primers are used in the study

Primer	Oligo sequence (5'-3')	Product size (bp)	Reference
PelA	F: CCTTCAGCCATCCGTTCTTCT	118	Colvin *et al.,* (17)
	R: TCGCGTACGAAGTCGACCTT		
PslA	F: CACTGGACGTCTACTCCGACGATAT	1119	Colvin *et al.,* (17)
	R: GTTTCTTGATCTTGTGCAGGGTGTC		
lasR	F: AAGTGGAAAATTGGAGTGGAG	130	Chowdhury and Bagchi, (18)
	R: GTAGTTGCCGACGACGATGAAG		
rhlI	F: TTCATCCTCCTTTAGTCTTCCC	155	Mukherjee *et al.,* (19)
	R: TTCCAGCGATTCAGAGAGC		
LecA	F: CGATGTCATTACCATCGTCG	215	Abdelraheem *et al.,* (20)
	R: TGATTGCACCCTGGACATTA		

Table 2-2
Thermal cycling conditions

Gene Name	Temperature (°C)/Time					Cycle Number
	First Denaturation	Condition of cycling			Last Extension	
		Denaturation	Annealing	Extensionn		
PelA	95/2 min.	95/15 sec.	45/60 sec.	72/45 sec.	72/7 min.	34
PslA	95/5 min.	94/30 sec.	55/30 sec.	72/1 min.	72/7 min.	30
LasR	95/2 min.	94/60 sec.	52/60 sec.	72/1 min.	72/10min.	30
rhlI	95/2 min.	94/60 sec.	52/60 sec.	72/1 min.	72/10min.	30
LecA	95/2 min.	95/60 sec.	53/60 sec.	72/90 sec.	72/10min.	30

Statistical Analysis

The results in the present study were evaluated statistically via T test and Chi square using Statistical Package for Social Sciences (SPSS) program version 23 at a probability of (P≤ 0.05) as a significant level between the parameters of the present study such as gender, age, and source of samples. *P* value of <0.05 was considered significant (21).

Results

Isolation and Identification of *P. aeruginosa*

A total of 800 samples were collected from patients suffering from urinary tract infections were admitted and visited five major hospitals in Al-Qadisiyah province, during the period between (November, 2020 to June, 2021), sixty (7.5%) isolates were showed positive and identified as *P. aeruginosa* by using selective media, biochemical test system and VITEK-2 compact system. During this study, the origin of *P. aeruginosa* isolates according to the gender, age and hospitalization are presented in Table (3-1). The number of *P. aeruginosa* isolates recovered from patients was 23 (38.34%), followed by 14 (23.34%), 10 (16.67%), 7 (11.66%) and 6 (10.0%) in the age group 16-30 years, 31-45 years, 46-60 years, less than 15 years, and more than 60 years respectively. The relation between the age groups wise profiles of *P. aeruginosa* infection was found statistically not significant (P= 0.081). Out of 60 patients from whom *P. aeruginosa* were isolated 38 (63.4%) were female patients and 22 (36.6%) were male patients which was statistically significant (*P*= 0.05) when compared to gender-distribution of patients from whom *P. aeruginosa* isolated. It was observed that hospitalized patients (24, 40.0%) were less infected with *P. aeruginosa* infection as compared to outpatients (36, 60.0%).

Table 3-1
Frequency distribution of patients with *P. aeruginosa* infections (n=60)

Patients profile	Status	No. (%) of samples (n=800)	No. (%) of *P. aeruginosa* (n= 60)	*P*-value
	<15	113 (14.13)	7 (11.66)	¥

Age group (year)	16-30	235 (29.37)	23 (38.34)	P=0.081 NS
	31-45	217 (27.12)	14 (23.33)	
	46-60	130 (16.25)	10 (16.67)	
	>60	105 (13.12)	6 (10.0)	
Gender	Male	330 (41.25)	22 (36.6)	¥ P=0.001 HS
	Female	470 (58.75)	38 (63.4)	
Hospitalization	Outpatient	513 (64.13)	36 (60.0)	¥ P= 0.640 NS
	Inpatient	287 (35.87)	24 (40.0)	

¥: Chi-square test; HS: Highly significant; NS: not significant at P ≤ 0.05

Sensitivity Profile of *P. aeruginosa* Isolates

All the 60 confirmed isolates of *P. aeruginosa* were evaluated for susceptibility to 17 selected antibiotics that are belonging to the ten generic classes of antimicrobial antibiotics according to (22). The Antibiotic susceptibility test was assessed by automated VITEK-2 compact system using AST-N222 cards. The sensitivity and resistance pattern of the isolates showed in (Table 3-2).

Table 3-2
Antibiotic susceptibility test of *P. aeruginosa* isolates by VITEK-2 compact system

Antibiotic agent	No. of isolates (n=60)			P-value
	Resistance (R)	Intermediate (I)	Sensitive (S)	
Piperacillin	45 (75.0%)	0 (0%)	15 (25.0%)	P= 0.001 HS
Ticarcillin	50 (83.34%)	0 (0%)	10 (16.66%)	
Ticarcillin/clavulanic	41 (68.34%)	0 (0%)	19 (31.66%)	
Piperacillin/tazobactam	15 (25.0%)	0 (0%)	45 (75.0%)	
Ceftazidime	40 (66.66%)	7 (11.66%)	13 (21.68%)	
Cefepime	39 (65.0%)	7 (11.66%)	17 (28.34%)	
Imipenem	24 (40.0%)	10 (16.66 %)	26 (43.34%)	
Meropenem	20 (33.34%)	9 (15.0%)	31 (51.66%)	
Ciprofloxacin	35 (58.34%)	14 (23.33%)	11 (18.33%)	
Pefloxacin	36 (60.0%)	3 (5.0%)	21 (35.0%)	
Amikacin	26 (43.34%)	7 (11.66%)	27 (45.0%)	
Tobramycin	28 (46.66%)	3 (5.0%)	29 (48.34%)	
Gentamicin	37 (61.66%)	5 (8.34%)	18 (30.0%)	
Aztreonam	33 (55.0%)	5 (8.34%)	22 (36.66%)	
Colistin	12 (20.0%)	0 (0%)	48 (80.0%)	
Minocycline	54 (90.0%)	2 (3.34%)	4 (6.66%)	
Trimethoprim+sulfamthoxazole	57 (95.0%)	0 (0%)	3 (5.0%)	

Molecular Detection of *LasR* and *RhlI* Genes:

The quorum sensing genomic *LasR* was detected in 56 of *P. aeruginosa* isolates (93.33%) gave positive results to this gene, these results achieved using specific *LasR* gene primers. which gave molecular length (130 bp), the results were shown in Figure (3-1). However, molecular detection of *RhlI* gene was done for isolates that previously detected as *P. aeruginosa*. The results showed that 56 of isolates (93.33%) gave positive results for this virulence gene. Positive results were detected by the presence of (155 bp) bands when compared with ladder as shown Figure (3-2).

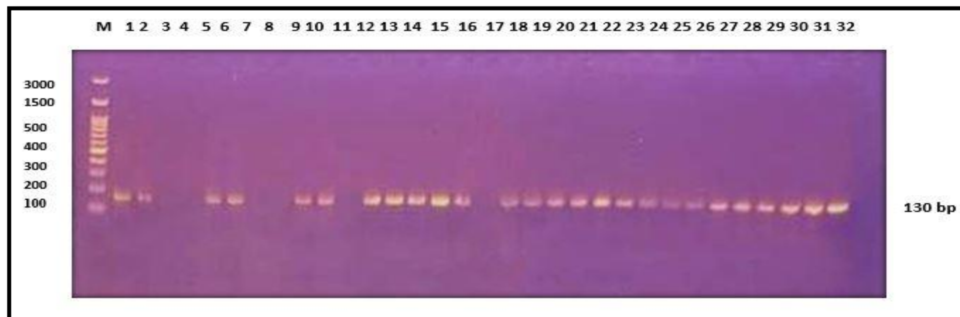

Figure 3-1. Ethidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of *LasR* gene (130 bp) for 1 hrs. at 70 volts, Lane M (Marker ladder 100-3000bp) Lanes (1, 2, 5, 6, 9, 10, 12, 13, 14, 15, 16, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32) Amplifying of *LasR* gene, Lanes (3, 4, 7, 8, 11, 17) No amplifying of *LasR* gene

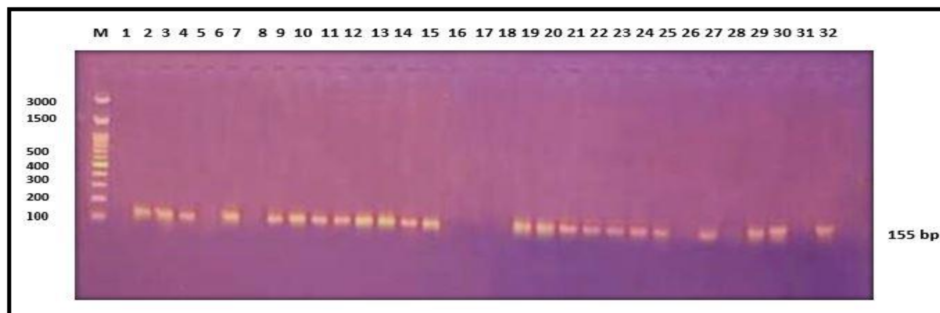

Figure 3-2. Ethidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of *RhI1* gene (155 bp) for 1 hrs. at 70 volts, Lane M (Marker ladder 100-3000bp) Lanes (2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 19, 20, 21, 22, 23, 24, 25, 27, 29, 30, 32) Amplifying of *RhI1* gene, Lanes (1, 5, 7, 16, 17, 18, 26, 28, 31) No amplifying of *RhI1* gene

Molecular Detection of *PelA, PslA* and *LecA* Genes

The *pelA, pslA and lecA* genes are essential for biofilm formation. They are also important for the up-regulation and production of virulence determinants. PCR was conducted over 60 isolates, using the *pelA*, *pslA* and *lecA* specific primers to

amplify these genes. The present results clarified the presence of *pelA* in 51 (98.07%) where are the bands appeared within the expected size of the gene (118bp) as shown in figure (3-3). While 50 (96.15%) of isolates have *pslA* gene, where are the bands appeared within the expected size of the gene (1119bp) as shown in figure (3-4).

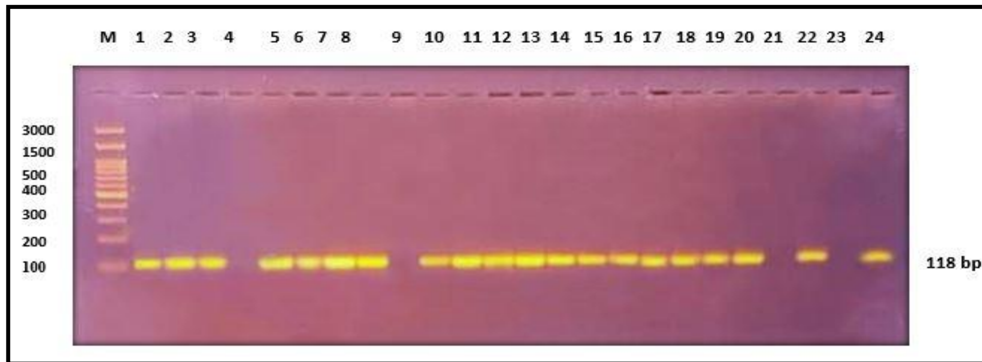

Figure 3-3. Ethidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of *pelA* gene (118 bp) for 1 hrs. at 70 volts, Lane M (Marker ladder 100-3000bp) Lanes (1, 2, 3, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 24) Amplifying of *pelA* gene, Lanes (4, 9, 21, 23), No amplifying of *pelA* gene

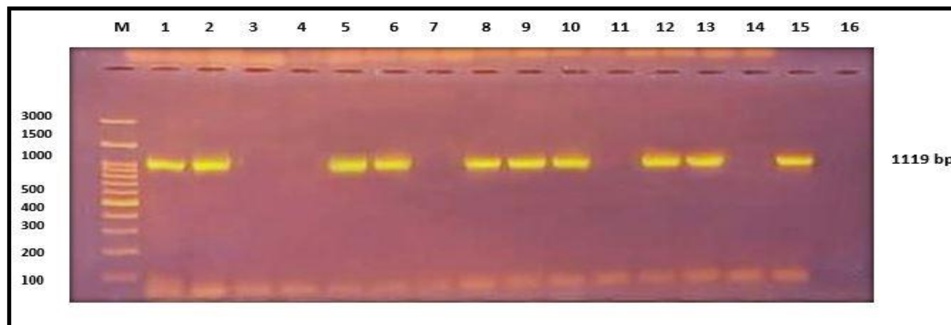

Figure 3-4. Ethidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of *PslA* gene (1119 bp) for 1 hrs. at 70 volts, Lane M (Marker ladder 100-3000bp) Lanes (1, 2, 5, 6, 8, 9, 10, 12, 13, 15) Amplifying of *PslA* gene, Lanes (3, 4, 7, 11, 14, 16) No amplifying of *PslA* gene.

The results also showed that the 48 (92.30%) of isolates have *lecA* gene where are the bands appeared within the expected size of the gene (215bp) as shown in figure (3-5).

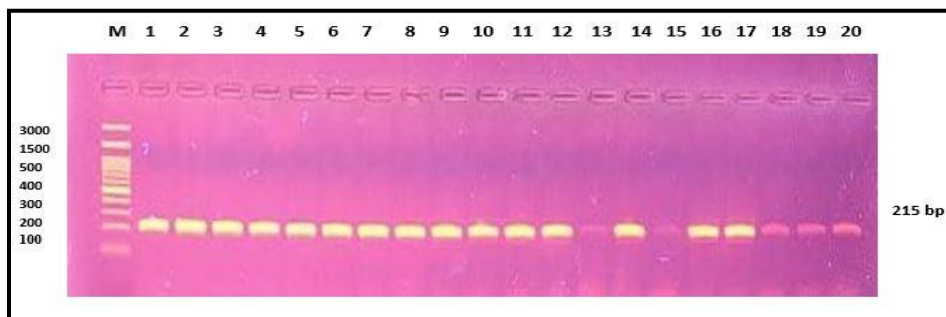

Figure 4-16. Ethidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of *lecA* gene (215 bp)for 1 hrs. at 70 volts, Lane M (Marker ladder 100-1500bp) Lanes (1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 14, 16, 17, 18, 19, 20) Amplifying of *lecA* gene, Lanes (13, 15), No amplifying of *lecA* gene.

Discussion

The present work aimed to study the capability of different *P. aeruginosa* clinical isolates to produce biofilm, antibiotic resistance of *P. aeruginosa* isolates and the association of biofilm production with the presence of *LasR, RhlI, PelA, PslA and LecA* genes. During a period of study, urine samples were collected from 800 patients with UTIs, out of which 7.5% patients had an established *P. aeruginosa* etiology. Belal, (23) in Najaf and Abdul-Wahid, (24) in Thi-Qar reported (9.8) and (8.9%) of *P. aeruginosa* among the urine samples of patients with UTI, respectively.

Resistance of *P. aeruginosa* to most antibiotics develops very rapidly and arises from the combination of unusually restricted outer-membrane permeability and secondary resistance mechanisms such as chromosomally or plasmid encoded periplasmic β-lactamase and energy- dependent multidrug efflux (25). In the current study, the highest antibiotic resistance of (75.0%, 83.34%, 90.0%, and 95.0%) was to piperacillin, ticarcillin, minocycline and trimethoprim+sulfamthoxazole, respectively. The moderate antibiotic resistance of (66.66%, 65.0%, 58.34%, 60.0%, 61.66%, and 55.0%) was to ceftazidime, cefepime, ciprofloxacin, pefloxacin, gentamicin and aztreonam, respectively. While, the lowest antibiotic resistance of (40.0%, 33.34% and 20.0%) was to imipenem, meropenem and colistin, respectively. A study in Iraq reported nearly similar results, where the resistant rates for penicillin antibiotics including ticarcillin, piperacillin and minocycline and trimethoprim+ sulfamthoxazole were 76.7%, 81.0%, 91.0% and 93.5%, respectively (26). Also, Abdul-Wahid, (24,40-51) in Iraq reported similar results where the resistant rates for ceftazidime, cefepime, ciprofloxacin, pefloxacin, gentamicin and aztreonam (64.0%, 63.5%, 59.0%, 58.5%, 63.0% and 57.0%), respectively. While, the other study in Iraq by Al-kazrage (27) reported the resistant rates for imipenem, meropenem and colistin (35.0%, 31.0% and 21.8%), respectively. The variation in the level of resistance between different studies may be attributed to the difference in geographical distribution, type and number of samples collected in each study and the difference in antibiotic policies implemented in each country.

The genomic *LasI* and *RhlI* were detected in 56 (93.33%) *P. aeruginosa* isolates that gave positive results to this genes, these results achieved using specific *LasI* and *RhlI* genes primers, which gave molecular length (130 and 155 bp), the results were shown in Figure (3-1, 3-2). These results were 7agreement with results obtained by Abdullah and Bunyan, (28) and Lahij *et al.,* (29) who found that, molecular detection of (QS) genes by used specific primers for *lasI* gene showed that (94.1% and 100%) of isolates were positive for both this genes in *P. aeruginosa,* respectively. *P. aeruginosa* form biofilms, matrix-enclosed multicellular accumulation that seems to supply increased existence capacity down different stress events. Quorum sensing (QS) has been shown to be implicated in the buildings of biofilm in various bacterial species. *P. aeruginosa* has two hierarchical QS systems known as las and *Rhl* (31). The *lasI* gene plays an important role in maintenance of *P. aeruginosa* biofilm, where that the signaling 3-oxo-C12-HSL (synthesized by *LasI*) is necessary for the establishment of *P. aeruginosa* biofilm, whereas a *lasI* mutant forms a flat and thin biofilm, and *lasI* is expressed in a large number of cells during the initial stage of biofilm formation (32). The present study confirm that important role of QS systems in Pathogenesis of *P. aeruginosa* bacteria and also indicated that *P. aeruginosa* able to causing clinical infections in humans despite an weakness of QS system in some isolates. On the other hand these results do not contradict with theory that QS system plays a main role in *P. aeruginosa* pathogenicity, and not all virulence factor controlled by QS (31). Li *et al*., (33) found that, most of *P. aeruginosa* isolates were positive for QS genes, also QS plays an important role in the pathogenesis of *P. aeruginosa* infection. Three factors are most important to induce biofilm: quorum sensing (QS), bis-(3′-5′)-cyclic diguanosine monophosphate (c-di-GMP), and small RNAs (sRNAs). *P. aeruginosa* has its own specific QS system (PQS) besides two common QS systems, *LasI–LasR* and *RhlI–RhlR*, in bacteria. PQS is interesting not only because there is a negative regulation from *RhlR* to *pqsR* but also because the null mutation in PQS leads to a reduced biofilm formation (34).

The results of the current study are in agreement with those of Maita and Boonbumrung, (35) who stated that the percentage of *pelA* was 95.0% while that of *pslA* was 90.1%, which were found in almost all clinical isolates of *P. aeruginosa*. Similar study observed that *pelA* gene was expressed heavily (90%) among biofilm-producing strains (36). However another study reported that the expression of *pelA* gene alone cannot predict whether the isolates can produce biofilm or not as they found that the expression of *pelA* gene among all studied *P. aeruginosa* isolates was (92.8%), irrespective of biofilm production (37). This was attributed to many factors controlling biofilm production. Kim *et al.,* (38) reported that *pelA* gene present in all biofilm-producing *P. aeruginosa* isolates. However, Al-Sheikhly *et al.,* (39) reported that *pelA* gene presents only 87.3% of the isolates. The high variability among the clinical isolates between different studies should be taken into consideration.

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