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Detection of *Lasb* and *Plch* Genes in *Pseudomonas Aeruginosa* Isolated From Urinary Tract Infections by PCR Technique

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Abstract:

Background: *Pseudomonas aeruginosa* is a Gram-negative bacterium, and it is a pathogen of opportunistic diseases that affects human with immunodeficiency or breaches of normal bodily defenses.

Aims: Isolation of *P. aeruginosa* bacteria from administration samples from patients with UTI, diagnosing this bacterium using enriched and selective culture media in addition to the biochemical assays. It also aims to extract bacterial DNA from isolates to investigate the *lasB* and *plcH* genes responsible for some virulence factors in *P. aeruginosa*, using the Polymerase chain reaction (PCR) technique.

Materials and methods: The current study included the collection of 477 samples of patients suffering from Urinary tract infections (UTIs) of both sexes, whose ages ranged between (19-65) years, for the period from September 1, 2020 to December 31, 2020, from several hospitals in the city of Baghdad.

Results: After the final diagnosis of the samples, 50 (10.48%) isolates of *P. aeruginosa* were obtained. The percentage of infection with this bacteria was higher in males than in females, 26 (52%) and 24 (48%), respectively. The highest rate of bacterial infection was in the age group (51-60) years, at 38%. The isolates were tested for antibiotic sensitivity using the Kirby-Bauer method, and the highest antibiotic resistance was Ceftazidime (98%), Aztreonam (96%), Amikacin (76%) and Tobramycin (74%). DNA was extracted from all bacterial isolates, and the polymerase chain reaction (PCR) was performed using specialized primers to investigate the *lasB* and *plcH* genes. The results showed that 24 (48%) isolates contain the *lasB* gene, and the resulting bundles were found to have a molecular weight (284 bp). The results also revealed that 24 (48%) isolates contain *plcH* gene with a molecular weight of the bundles (608 bp).

Conclusion: it was found in the current study that the bacterial isolates that contained the *lasB* gene are the same as that containing the *plcH* gene, so the possibility of these isolates for pathogenicity is higher than if they contained only one of the two genes.

Key words: *Pseudomonas aeruginosa*, *lasB* gene, *plcH* gene, Urinary tract infections (UTIs).

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Introduction:

Pseudomonas aeruginosa is a Gram-negative bacterium, from the family of Pseudomonadaceae, that is ubiquitous and is able to survive in a wide range of environments (Silby et al., 2011). This bacterium is one of the pathogens of opportunistic diseases that affect people who are immunocompromised or who suffer from breaches of normal bodily defenses. It is a pathogen that can infect all human tissues and organs such as the urinary tract, respiratory system, bones, joints, soft tissues (burns) and dermatitis (Gellatly and Hancock, 2013).

Urinary tract infections UTI is one of the most common infections spread around the world. It is the most common reason for the use of antibiotics in Europe and America (Abbo and Hooton, 2014). Although the incidence of urinary tract infections caused by *P. aeruginosa* is lower than that of *Escherichia coli* and *Klebsiella pneumoniae*, however, complex urinary tract infections as well as catheter-related and hospital conditions are more frequent in *P. aeruginosa* than the mentioned species. (Ironmonger *et al*., 2015).

P. aeruginosa possesses a wide range of virulence factors that facilitate infection and colonization of host cells (Newman *et al*., 2017; AL-Rubaye *et al*., 2020; AL-Rubaye *et al*., 2020). In addition, some virulence factors in *P. aeruginosa* are responsible for some genes, including the *lasB* gene that encodes the enzyme *lasB* elastase. This enzyme has an active role in protein degradation and necrosis processes (Shuwaikh, 2016), it is also a highly toxic agent that causes tissue damage and invasion (Cathcart *et al*., 2011). *P. aeruginosa* also possesses the *plcH* gene that encodes for the phospholipase enzyme, which is an important virulence factor because it helps pathogens invade the host's cell and modify the phospholipid content in the host's cell membrane, and it is also essential in assisting bacteria in the host's immune evasion mechanism (Bandana *et al*., 2018). Therefore, the aims of this study are to isolate *P. aeruginosa* from administration samples from patients with UTI and diagnose this bacterium using rich and selective culture media and biochemical assays. It also aims to extract the bacterial DNA from the isolates of this bacterium, and to investigate the *lasB* and *plcH* genes, which responsible for some of the virulence factors in this bacterium, using the Polymerase chain reaction (PCR) technique.

Materials and Methods:

Samples collection: This study included the collection of 477 urine samples from patients with urinary tract infection of both sexes, ranging in age from (19-65) years, for the period from September 1, 2020 to December 31, 2020, from the Teaching Laboratories Hospital, the Specialized Surgery Hospital, and the Hospital Baghdad Education Department of the City of Medicine. In addition to Al-Karamah Teaching Hospital and Al-Yarmouk Teaching Hospital.

Identification of bacterial isolates: The isolates of *P. aeruginosa* were diagnosed and confirmed by using these methods as follows:

Cultivation of samples: Blood agar, MacConkey agar medium, Cetrimide agar were used to study the phenotype of colonies of *P. aeruginosa*. King A medium, King B medium, were prepared according to the instructions of company (Himedia, India) and (Mortimer *et al*.,

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1981) to isolate and diagnose the bacteria under study. Gram stain was also used to diagnose *P. aeruginosa* in samples (Baron *et al*., 2007).

Biochemical tests: The Catalase test, the oxidase test, and the IMVIC test were used (Collee *et al*., 1996; Prescott, 2002).

Motility test: Motility medium was used to perform this test (Collee *et al*., 1996).

Grown at 42 C° test: This test was performed using nutrient agar plates which inoculated with *P. aeruginosa*, then the plates were incubated at 42 °C for 18-24 hours, the positive result was growth at this temperature (Collee *et al*., 1996).

API 20E system: API 20E diagnostic kit (BioMerieeux, France) is approved to perform confirmatory diagnosis of bacterial isolates.

Staining of urine deposits: The method for dyeing urinary sediment was conducted according to the two sources (Rodriguez *et al*., 2011; Harris 1969).

Antimicrobial susceptibility test: Disk diffusion method was used on Muller-Hinton medium after culture with isolates of *P. aeruginosa* to test for antibiotic sensitivity.

DNA Extraction: The bacterial DNA was isolated from the isolates of P. aeruginosa in culture media according to the method of operation of the extraction kit (ZR Fungal/ Bacterial/ Yeast DNA MiniPrep^{TM}, Catalog No. D6005).

Stock Solution of primer preparation: The initiator solutions were prepared according to the manufacturer's instructions (Integrated DNA Technologies; IDT, Canada), to obtain a final concentration of 100 pmol/ µl as a stock solution.

10 Pmol/µl primer solution preparation: The solution was prepared by adding 10 µl of the storage solution to 90 µl of ionic distilled water (ddH_2O), then they were mixed well with a Vortex mixer to homogenize the solution before use, after which the solution was kept at a temperature of -20 °C until use (Table 1).

Table (1): primer sequences used in current study

genes	Primer sequence(5'-3')		Product size (base pare)
lasB	F	5'- GGAATGAACGAAGCGTTCTCCGAC - 3'	284 (1)
	R	5'- TTGGCGTCGACGAACACCTCG - 3’	
plcH	F	5'- GCACGTGGTCATCCTGATGC - 3'	608 (2)
	R	5'-TCCGTAGGCGTCGACGTAC - 3’	

(1): Al-Shimmary, 2020; (2): Faraji *et al*., 2016.

Determination of extracted bacterial DNA concentration and purity: The concentration and purity of the bacterial DNA extracted from isolates of *P. aeruginosa* was determined according to the source (Nakayama *et al*., 2016) using a Nanodrop device.

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Polymerase chain reaction (PCR) for genes detection: The reaction mixture was prepared using a Maxime PCR preMix kit (i-Taq) 20 lrxn and according to the manufacturer's instructions (iNtRON) as in Table (2):

Table (2): Optimum conditions for PCR to detect for *lasB* and *plcH* genes

No.	phase	Temperature (°C)	Time	No. of cycle
1	Initial DNA Denaturation	95	5 min.	1
2	DNA Denaturation	95	45 sec.	35
3	Annealing	60	45 sec.	
4	Extension	72	45 sec.	
5	Final extension	72	7 min.	1

Detection of amplified DNA bands: The presence of amplified DNA packages was investigated according to the source (Al-Ahmadi and Roodsari, 2016).

Results and discussion:

The current study included the collection of 477 urine samples from both sexes, males and females, who suffer from urinary tract infection. After the final diagnosis of the samples, 50 (10.48%) isolates of *P. aeruginosa* were isolated. The incidence of infection with *P. aeruginosa* was higher in males than in females, 26 (52%) and 24 (48%), respectively. The highest rate of bacterial infection was within the age group 51-60 years, followed by the age group 50-41 years, 19 (38%) and 11 (22%), respectively table (3).

Table (3): Age distribution of *P. aeruginosa* in patients with urinary tract infections

Age groups (year)	Number of *P. aeruginosa* isolates		Total (%)
	Male (%)	Female (%)	
1-10	(0) 0	(0) 0	(0) 0
11-20	(3.84) 1	(4.16) 1	(4) 2
21-30	(7.69) 2	(8.33) 2	(8) 4
31-40	(11.53) 3	(8.33) 2	(10) 5
41-50	(23.07) 6	(20.83) 5	(22) 11
51-60	(38.46) 10	(37.5) 9	(38) 19
61-70	(15.38) 4	(20.83) 5	(18) 9
Total (%)	(100) 26	(100) 24	(100) 50

The results of the current study are close to the results of researchers Al-Salamy and Al-Hilli, 2012, where they found that the isolation rate of *P. aeruginosa* from urine samples in Baghdad was 26 (14.99%) (Al-Salamy and Al- Hilli, 2012). The percentage of isolate bacteria in the current study was 10.48%. In other local studies, the percentages varied from one study to another. Al-Hashemi in 2020 (Al-Hashemi, 2020) indicated that the percentage of isolation of *P. aeruginosa* from urine samples was 27.27%. Nader and others in 2017 (Nader and others, 2017) were find that 4 of the 11 urine samples were containing *P. aeruginosa*, at a rate of 36.36%, which is higher than the results of the current study. The reason for this difference

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is that local studies focused on investigating *P. aeruginosa* from isolates taken from a variety of sources (urine, wounds, burns, etc.). While the current study focused on isolating *P. aeruginosa* from urine samples only. For the purpose of comparison, only the percentage of presence of *P. aeruginosa* in urine samples was calculated from other researches, and it was noticed that the number of urine samples was small in these studies and that gives a result which statistically inaccurate regarding the local presence of bacteria.

In the current study, urine samples were cultured on selective and special culture media to isolate *P. aeruginosa* after performing the general urine examination - GUE of the urine samples. Urine samples were selected for culture and showed a positive result for the leukocyte esterase (LE) test when using Rapid urine dipsticks. Results from this study showed that urine samples with LE test positive and pus cells higher than 5 cells / microscopic field gave positive results on diuretic transplantation. This is consistent with numerous international studies (Simerville *et al*., 2005; Laosu-angkoon, 2013; Dadzie *et al*., 2019).

The diagnosis was based on the form of colonies formed by isolates of developing bacteria on the enriched, special and selective culture media mentioned in the materials and methods of work. Where colonies of *P. aeruginosa* bacteria appeared on selective media (MacConkey agar) in pale yellow color, because they are not fermented by the non-lactose fermenter, figure (1). These results are consistent with the results of previous research (Forbes *et al*., 2002).

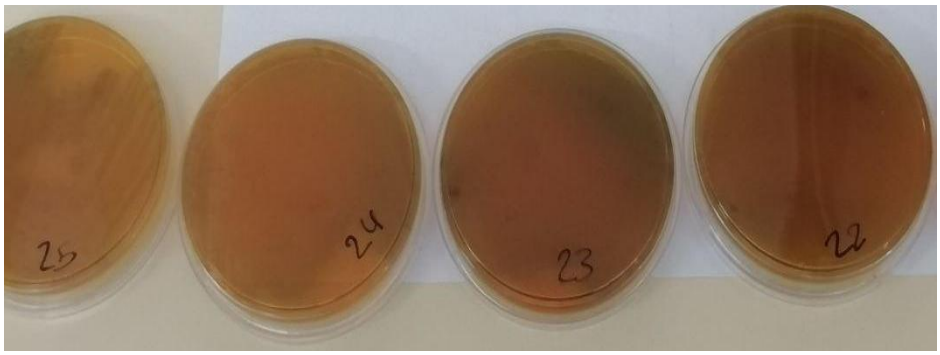

Figure (1): Colonies of P. aeruginosa isolates on the selective culture medium MacConkey agar appear pale yellow.

On the enriched media (blood agar), the bacteria gave-hemolysis colonies, which is evidence of the ability of bacteria to produce hemolysin, which analyzes red blood cells on the culture medium. Whereas the growth of the bacterial colonies' characteristics on the nutrient agar media was distinguished according to their production of dyes and the smell similar to the smell of grapes (Selim *et al*., 2015). It was also found that the bacteria grew on the nutrient media when incubated at a temperature of 42 ° C for a period of 24 hours. More densely than it is with a degree of 4 ° C, figure (2), and this trait is a diagnostic feature that distinguishes *P. aeruginosa* from the rest of the species of the genus Pseudomonas (MacFaddin, 2000).

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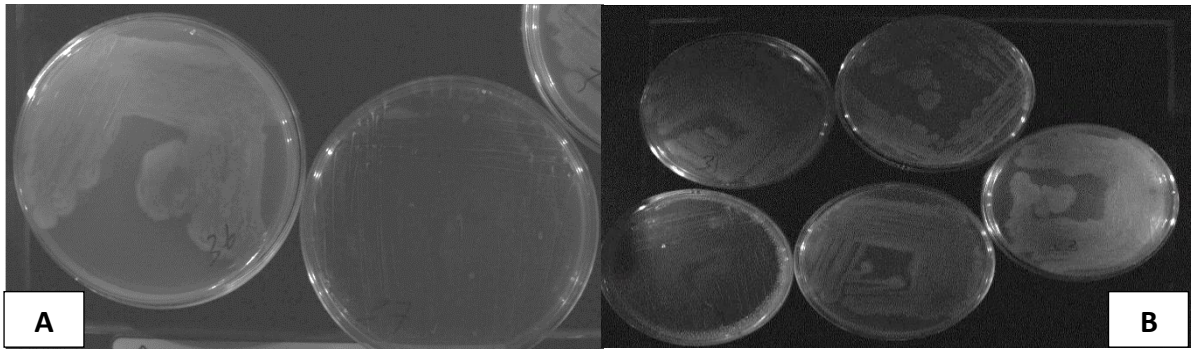

Figure (2): A and B, the density of the growth of colonies of P. aeruginosa isolates on nutrient agar at 42 ° C more than at 4 ° C.

When it comes to the growth of bacteria on cetrimide agar medium, the bacterial colonies appeared greenish-yellow, and these results are consistent with other studies (Tang and Stratton, 2006; Al-Dahmoshi, 2013). As well, the bacterial colonies on King A agar media produced the blue and green pyocyanin medium, while on King B agar medium, all isolates did not produce the biocyanin stain (Blondel-Hill *et al*., 2007).

Using a regular light microscope, microscopy reveals that *P. aeruginosa* is a Gram-negative bacillus, and these results are consistent with other studies (Al-Dahmoshi, 2013; Diggle and Whiteley, 2020). Biochemical tests showed that all bacterial isolates gave a positive result for the catalase test, and bacterial isolates showed a positive result for the oxidase test, and these results were consistent with the results of previous studies (Tadess and Alem, 2006; Al-Daraghi and Al-Badrwi, 2020).

As for the IMViC test, which included the Indole test, the Methyl red test, and the Vogas-proskauer test, results were negative for these three tests. While the results of the citrate utilization test were positive, and the motility test also showed a positive test result, as proven by previous researchers in this field (Rundell *et al*., 2020). The Urease test also showed a variable result between positive and negative. These results are consistent with many previous studies (Tades and Alem, 2006; Todar, 2011). As for the diagnosis of the Api 20E system, which includes 20 tests, the results were interpreted based on the "Api 20E Analytic Profile Index" and the results showed that 50 isolates belong to *P. aeruginosa*.

The sensitivity of bacteria to antibiotics was tested for ten antibiotics, and the results of the current study showed that all *P. aeruginosa* isolates showed a clear difference in the antibiotic resistance used in this study, see table (4):

Table (4): Number of isolates and percentage of antibiotic resistance of *P. aeruginosa:*

Group	Antibiotics	Code	*P. aeruginosa* isolates		
			R (%)	I (%)	S (%)
Aminoglycosides	Tobramycin	TOB	37 (74)	2 (4)	11 (22)
	Amikacin	AK	38 (76)	5 (10)	7 (14)
Carbapenems	Imipenem	IPM	24 (48)	4 (8)	22 (44)
	Meropenem	MEM	31 (62)	4 (8)	15 (30)
Cephalosporins	Ceftazidime	CAZ	49 (98)	--	1 (2)
Quinolones	Levofloxacin	LEV	11 (22)	11 (22)	28 (56)

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	Ciprofloxacin	**CIP**	**15 (30)**	**20 (40)**	**15 (30)**
	Norfloxacin	**NOR**	**24 (48)**	**11 (22)**	**15 (30)**
Monobactams	**Aztreonam**	**ATM**	**48 (96)**	**2 (4)**	**--**
Penicillins	**Piperacillin**	**PRL**	**25 (50)**	**21 (42)**	**4 (8)**

R: Resistance, I: Intermediate, S: Sensitive

DNA was extracted from 50 isolates of *P. aeruginosa* growing on nutrient agar medium, according to the instructions of the provider company ZR Bacterial DNA MiniPrepTM D6005. The purity and concentration of the DNA extracted in each bacterial isolate were measured using Nanodrop spectrophotometer system. The concentration of the bacterial DNA of all the isolates was between (55-298) ng / µL, this concentration is sufficient for use in DNA amplification by PCR technique. DNA purity (measured by the absorbance reading at wavelength 260/280 nm) for all bacterial isolates ranged between (1.56 -2). The results of the current study are close to the results of the researcher Al-Azzawi (Al-Azzawi, 2018), as the concentration of DNA samples in her study ranged between (39.8 - 264.5) ng / µL, and the range of purity ranged between (0.84-1.94). As for the results of the study by researcher Al-Shamaa (Al-Shamaa, 2016), the purity of the DNA of the bacterial isolates ranged between (1.8 – 2), and the DNA concentration in the samples ranged between 60-110 ng / µl. Figure (3).

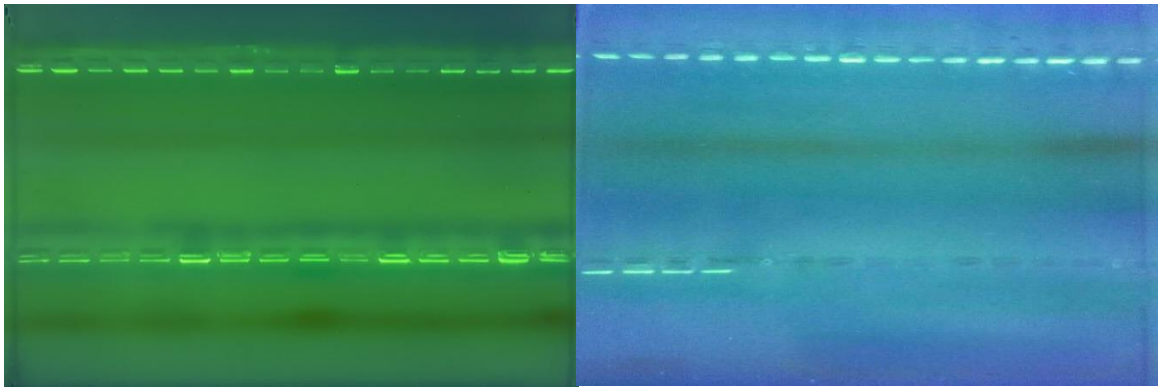

Figure (3): A and B: Gel electrophoresis of genomic DNA extracted from 50 isolates of *P. aeruginosa*, 1% agarose gel with a current of 70 V / cm^2 for 30 minutes

In the current study, the *lasB* and *plcH* genes possessed by *P. aeruginosa* were detected using a thermocycler for PCR to amplify the DNA according to the conditions required for the thermal cycling device. PCR was performed for all bacterial isolates using specialized primers to detect these two genes. The results showed that 24 (48%) isolates of these bacteria contain the *lasB* gene, and when comparing the amplified bundles with the volume band index of the DNA ladder, the resulting DNA bundles were found to have a molecular weight (284 bp) as shown in the figures (4A and B). The results also showed that 24 (48%) isolates of the bacteria under study contain the *plcH* gene. When comparing the amplified bundles with the size range index of the DNA ladder, it was found that the resulting DNA bundles have a molecular weight (608 bp) as shown in the figures (5 A and B).

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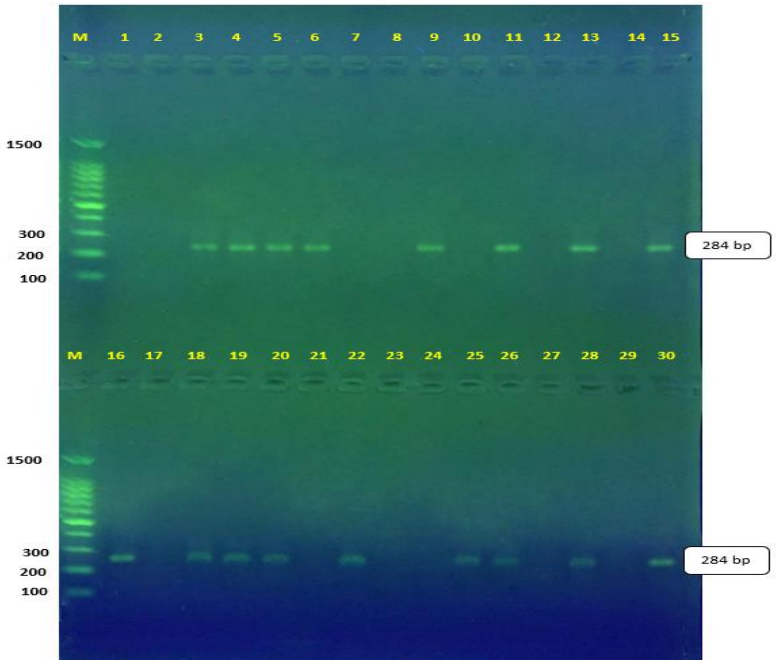

Figure (4 A): Electrophoresis of the polymerase chain reaction products for *lasB* gene, the band size is 284 bp. The product was electrophoresed on 2 % agarose at 70 volt / cm^2 for 60 min. M: DNA ladder marker (100-2000bp). The lines 3, 4, 5, 6, 9, 11, 13, 15, 16, 18, 19, 20, 22, 25, 26, 28, and 30 are the *P. aeruginosa* isolates having *lasB* gene.

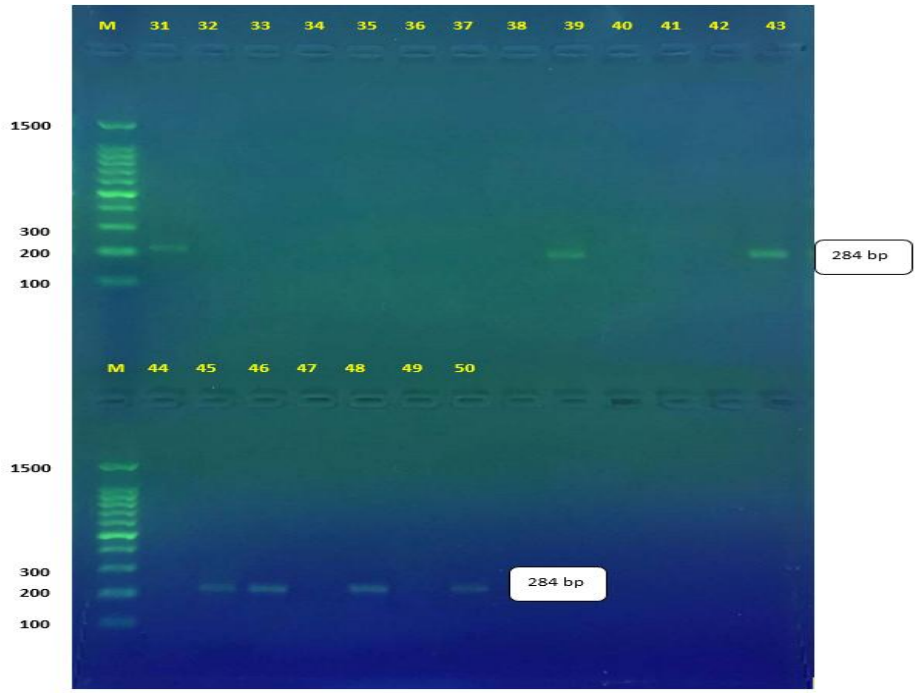

Figure (4 B): Electrophoresis of the polymerase chain reaction products for *lasB* gene, the band size is 284 bp. The product was electrophoresed on 2 % agarose at 70 volt / cm^2 for 60 min. M: DNA ladder marker (100-2000bp). The lines 31, 39, 43, 45, 46, 48, and 50 are the *P. aeruginosa* isolates having *lasB* gene.

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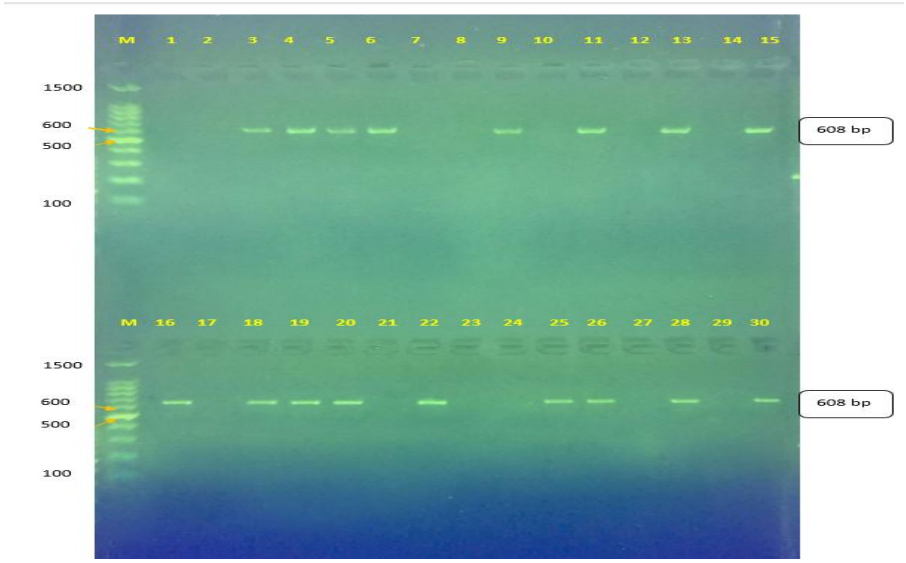

Figure (5 A): Electrophoresis of the polymerase chain reaction products for *plcH* gene, the band size is 608 bp. The product was electrophoresed on 2 % agarose at 70 volt / cm^2 for 60 min. M: DNA ladder marker (100-2000bp). The lines 3, 4, 5, 6, 9, 11, 13, 15, 16, 18, 19, 20, 22, 25, 26, 28, and 30 are the *P. aeruginosa* isolates having *plcH* gene.

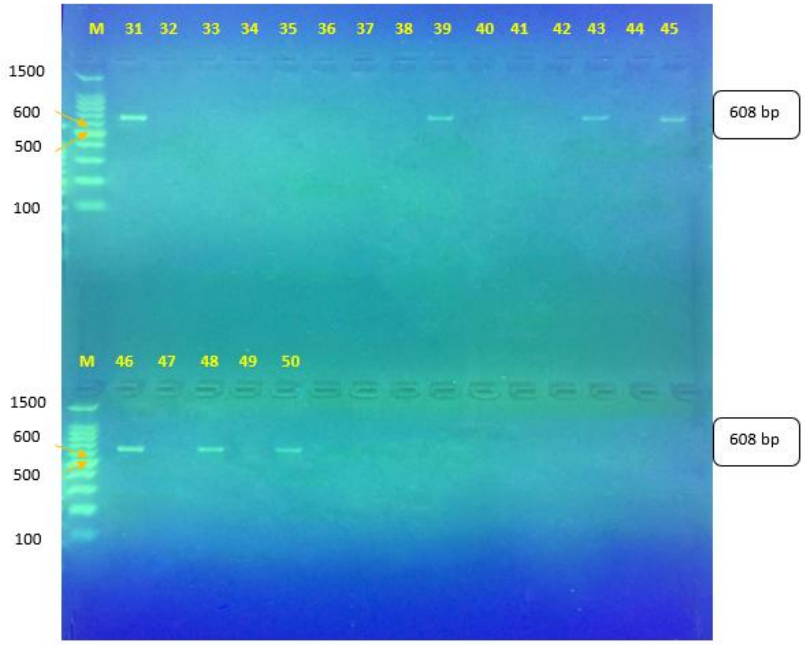

Figure (4 B): Electrophoresis of the polymerase chain reaction products for *plcH* gene, the band size is 608 bp. The product was electrophoresed on 2 % agarose at 70 volt / cm^2 for 60 min. M: DNA ladder marker (100-2000bp). The lines 31, 39, 43, 45, 46, 48, and 50 are the *P. aeruginosa* isolates having *plcH* gene.

In local and international studies, Al-Arnaouti found that the percentage of urine isolates possessing the *lasB* gene is (62.5%) (Al-Arnaouti, 2015), while the researchers, Raouf and Tawfiq, found that the percentage of urine isolates that possess the *plcH* gene is 66.7% (Raouf and Tawfiq, 2014). In international studies, a group of researchers found that the percentage of urine isolates that contain *P. aeruginosa* possessing the *lasB* and *plcH* genes is 75% each (Sabharwat *et al*., 2014). On the other hand, other researchers found in 2017 that the percentage of *P. aeruginosa* that possesses the genes *lasB* and *plcH* was 24% and 18.5%, respectively (Waheed Ullah *et al.*, 2017). However, it is noted that the sample size was 54 Isolation, but the study did not clarify the number of urine isolates that were used.

Conclusion: it was found in the current study that the bacterial isolates that contained the *lasB* gene are the same as that containing the *plcH* gene, so the possibility of these isolates for pathogenicity is higher than if they contained only one of the two genes.

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