Regulate the Risk Factors of Pseudomonas aeruginosa by some of Sigma Factor Genes

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KEYWORDS

Pseudomonas aeruginosa, Sigma factor Genes , infection

ABSTRACT

In this research, a study was conducted isolating and identification of *Pseudomonas aeruginosa* bacteria from clinical samples from various pathological conditions such as burns , otitis media , urinary tract infection , wounds, eye infection, blood infection, catheter infection, throat , vaginal infection and surgical. The present study includes 313 samples collected from patients who attend Alsader Medical City and Al- Hakim General Hospital during the period extended From September 2023 to December 2023 (men and women) with age groups between (1 -77) years.

The identification of bacterial isolates were detected by classical and molecular technique (PCR), were the frequency among males 163 (52.07%) was more than that in female 150 (47.92). The samples distribution is made according age group , it appears high for the following high (15.97) and (12.77) with group (16-30) years of male and female respectively .

The results revealed that 57/313 specimens of the total number of samples are *Pseudomonas aeruginosa* by identified according to their morphological, physiological and biochemical properties. Also with molecular identification by using four primers wre the results showed *16S rRNA* gene was detected in 57/57 (100%) *P. aeruginosa* isolates , the *Las biofilm* gene was detected in 43/57 (75.43%) , the *algD* gene was detected in 41/57 (71.92%) and 41/57 samples show positive results with the *oprL* gene. Six types of antibiotics were also tested to determine the resistance of the samples to them.

1. Introduction

The most frequent gram-negative bacterium that isn't related to the Enterobacteriaceae family is *Pseudomonas aeruginosa*. It is typically found in damp environments in hospitals and is extensively spread in nature. It may infect people with aberrant host defenses and colonize healthy individuals in whom it is saprophyte (Diggle & Whiteley, 2020).

P. aeruginosa is the cause of community-acquired infections in the following areas: skin (folliculitis); external ear canal (otitis externa); eye, after trauma; bone (osteomyelitis); heart (endocarditis) in IV drug users; and respiratory tract (cystic fibrosis CF patients) (Rodriguez, et al., 2020; Morin, et al., 2021).

The central nervous system, bloodstream (bacteremia), wounds, respiratory tract, and urinary tract are among the hospital-acquired illnesses. Exotoxins, endotoxins, proteases, phospholipases, rhamnolipids, and the blue-green pigment pyocyanin an alginate-like exopolysaccharide that causes the mucoid phenotype as well as pili and intrinsic resistance to numerous antimicrobial agents are among P. aeroginosa's virulence factors (Al - Ammar ,*et al* 2012:Soberon, *et al.*, 2021).

The main component of gram-negative bacteria's outer membrane is lipopolysaccharide (LPS), which allows the bacterium's entrance into eukaryotic cells and shields the pathogenicity of the bacteria from host defenses. Bacteria secrete many virulence factors that help them invade, colonize, cause tissue damage, invade the bloodstream, and spread to various areas of the body (Fisher and Mobashery. 2020 ; Pussinen, et al., 2022) .

Bacteria produce many proteases, such as: elastase, alkaline protease, protease IV, and staphylolytic protease, all of which are important virulence factors. Elastase is responsible for severe tissue damage during Bacterial infection of humans. Basal protease has the ability to destroy many tissue components, such as protein elements in connective tissue, and this indicates the role that this enzyme plays in invading and decomposing

tissues. Protease IV is one of the virulence factors that allows bacterial pathogenicity in keratitis (O'Callaghan, et al., 2019; Tsavea, et al., 2023)

2. Methods:

Collection of specimens :

From September 2023 to December 2023, (313) specimens were collected from patients infected with *P. aeruginosa* in Al-Sader Teaching Hospital and Al- Hakim General Hospital, The collected specimens were inoculated on MacConkey agar then incubated at 37 °C for 24 hr. The pale non lactose fermentor colonies were selected then a single colony was inoculated on Blood agar for the activation and detection of bacterial ability to lyses red blood cells (β-hemolysis). The isolated colonies were inoculated on Cetrimide 0.03% medium, incubated at 37°C for 24 hr. Then a single colony from colonies that grow on Cetrimide media was inoculated on King A agar and King B agar to determine their ability to produce pigments. Single colonies were inoculated on Nutrient agar to carry out other biochemical tests that confirmed the identification of bacterial isolates.

Antibiotic Susceptibility Test Disk Diffusion Method:

It was conducted using a pure culture of the bacterial organism previously described, according to the Clinical Laboratory Norm Institute (CLSI, 2023).

The samples were tested on 6 types of antibiotics to determine their degree of resistance and sensitivity, as shown in the results.

Molecular detection of *P. aeruginosa*

The DNA extract of bacterial isolates were subjected to four primers genes listed in Table (1) by using PCR. The protocol was used depending on Promega Biosystem manufacturer's instruction. Single reaction (final reaction volume 20 µl) as in table (2). All PCR components were assembled in PCR tube and mixed by refrigerated microcentrifuge at 50 rcf for 10 second.

Table (1): Primers used in this study

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Reference
16S RNA	F : GGGGATCTTCGGAACCTCA R : TCCTTAGAGTGCCCAAACCCG	956	(Hussein, *et al*., 2023)
LasR	F :CTGTGGATGCTCAAGGACTAC R :AACTGGTCTTGCCGATGG	133	(Bahari, *et al*., 2017)
***oprL* PAL**	F: ATG GAA ATG CTG AAA TTC GGC R:CTT CTT CAG CTC GAC GCG ACG-3	504	(Xu *et al*., 2004)
algD	F:CTACATCGAGACCGTCTGCC R: GCATCAACGAACCGAGCATC	593	(Banar, *et al*., 2016)

Table (2) : Protocol of monplex PCR reaction mixture volumes:

Mixture solution	
Master mix	8µL
DNA template	5µL
Forward primers	1.5µL
Reverse primers	1.5µL
Deionidied water (d d water)	4µ L
Final volume 20 µL	

3.Results and discussion :

During a six- month period, from 1st September 2023 to February 2024 a total of 313 clinical specimens including burns (170), otitis media (24), urinary tract infection (49), wounds (39) , eye infection (7) , blood infection (5) , catheter infection (8) , throat (4) vaginal infection (3) and surgical (4) ,were collected. All specimens were cultured on MacConkey agar and blood agar , as in Figure (1).

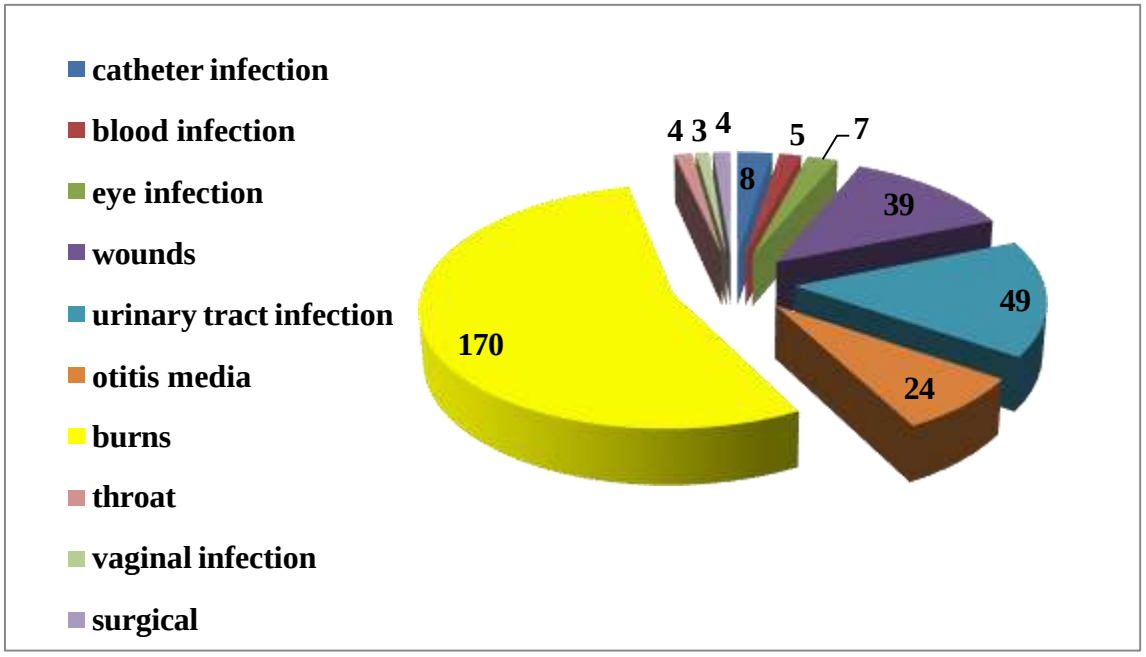

Figure (1): The distribution of samples according to their type.

With clinical specimens at patients age range between (1-77 years). It was shown that 313 samples were distributed accordingly age, the lowest incidence was in the age groups over (61-77) 9.90 % , and the highest incidence was the (16-30) age group (28.75 %), as in Table (3).

Table (3): The distribution of patients according to age groups and sex.

Sex	Age group No. (%)					
	1-15	16-30	31-45	46-60	61-77	total
Female	35 (11.18)	40 (12.77)	36 (11.50)	24 (7.66)	15 (4.79)	150 (47.92)
Male	38 (12.14)	50 (15.97)	29 (9.26)	30 (9.58)	16 (5.11)	163 (52.07)
Total	73 (23.32)	90 (28.75)	65 (20.76)	54 (17.25)	31 (9.90)	313 (100 %)

Regarding the sources of the isolates and their percentage of presence in each source, which, as we mentioned, were (57) clinical samples from the *P. aeruginosa* of isolation (burns (14), otitis media (11), urinary tract infection (7), wounds (11) , eye infection (2) , blood infection (3) , catheter infection (4) , throat (1) vaginal infection (2) and surgical (2)), the number of *P. aeruginosa* bacteria isolates among them was (57/313) samples, with an isolation rate of (38.77)%. As in Table (4).

Table (4): Isolation and their percentage of presence *P. aeruginosa* in each source.

Sample source	Number of samples	Number of isolates	Percentage of isolation
Burns	170	14	24.56 %
Otitis media	24	11	19.29 %
Urinary tract infection	49	7	12.28 %
Wounds	39	11	19.29 %
Eye infection	7	2	3.50 %
Blood infection	5	3	5.26 %
Catheter infection	8	4	7.01 %
Throat	4	1	1.75 %
Vaginal infection	3	2	3.50 %
Surgical	4	2	3.50 %
Total	313	57	100 %

Identification of *P. aeruginosa* isolates:

An early step of identification, different local isolates were examined according to their ability to staining, shape, color, size, production of pigments, transperancy and mucoidal properties after plating on nutrient agar and it was explained by Palleroni (2008). All the bacterial isolates were examined for gram stain, Oxidase test, Catalase test, Growth in 42°C and 4°C, IMViC test, Kligler iron test, Oxidative-Fermentation test, Motility test, Urease production test and Hemolysin production test, in table (5) displays the results that appeared in tests of the studied samples (Huchaimi ,*et al* 2018).

Table (5): Diagnostic and biochemical tests for *P. aeruginosa*

Tests	Result
MacConkey agar	Growth (pale colonies)
Blood agar	β-hemolysis
cetrimide agar	)+(
Growth at 4 °C	)-(
Growth at 42°C	)+(
King A medium	Growth + secretion of the pigment pyocyanin
King B medium	Growth + secretion of fluorescein dye
Oxidase	)+(Purple color
Catalase	)+(Buzzing and bubbling
Indole test	)-(
Methyle red	)-(
Vogas proskauer	)-(
Citrate utilization	)+(
TSI	-- K / K
Oxidative-fermentation medium	)+(
Motility	)+(

Resistance of isolates to antibiotics:

The sensitivity of (57) isolates of *P. aeruginosa* bacteria to (6) antibiotics was tested table (6), which are: Amikacin, Ceftazidim, Cefotaxim, Augmentin, Ampicillin and Tetracyclin by using the Kirby-Bauer method, and vitek2 system. The sensitivity or resistance of bacteria to the antibiotic was determined by measuring the diameters of the inhibition zones around the disc (CLSI, 2023). This test is used for the purpose of obtaining the resistance pattern (antibiogram) of the isolates under study.

Table (6) : Percentages of resistance in *P. aeruginosa* isolates to some antibiotics.

Antibiotic / MIC	Number of resistant isolates n/ 57	Percentage %
Cefotaxim (30)	29	50.87%
Amikacin (30)	57	100%
Ampicillin (10)	57	% 100
Augmentin (30)	57	% 100
Tetracyclin (30)	43	75.43%
Ceftazidin (30)	18	31.57%

It is clear from Table (6) that the percentage of resistance of *P. aeruginosa* bacteria to the antibiotics Ampicillin , Amikacin and Augmentin was 100%. This is consistent with the results obtained by Urban-Chmiel, (2022), as the percentage of resistance to the antibiotics Ampicillin was 100%, while Al Yousef , (2016) reported that the resistance rate of *P. aeruginosa* bacteria to fully resistant to tetracycline, while khalid, (2023) reported that the resistance rate to Ampicillin was 98.6 % , As for Ahmed, *et al*., (2020) were resistance rate to Augmentin and Ampicillin was (95%, 95%) respectively .

The high resistance to this group of antibiotics is due to incorrect use and incorrect doses, which generated resistance in bacteria to these antibiotics, as AL-Kraety , *et al*., (2007)sueh, *et al*., (2005) found that the increase in bacterial resistance to a number of antibiotics, including β-lactam antibiotics, increases with the increase in consumption of these antibiotics(Al-Muhanna *et al* 2020)..

This resistance is also due to the bacteria's production of a large number of β-lactamase enzymes that destroy these antibiotics (Eiamphungporn, *et al*., 2018),

Pandey *et al*., (2005) found in a study conducted in India, 36% of *P. aeruginosa* isolates produced β-lactamase enzymes, and in a study conducted in Brazil, 77% of isolates produced the MBL (Metalo - Beta - Lactamase) enzyme (Tan, *et al*., 2021).

The genes for β-lactamase enzymes are carried either on a transmissible plasmid , or they may be carried on the chromosome (Hussain, *et al*., 2021).

The efflux pump system also plays an important role in bacterial resistance to beta-lactam antibiotics, even those that do not produce the β-lactamase enzyme (Lorusso, *et al*., 2022).

The results showed that the antibiotics Amikacin and Gentamycin, within the group of aminoglycoside antibiotics, had good effectiveness against *P. aeruginosa* bacteria, as the resistance rate was 3.50% and 19.29%, respectively. This is consistent with the results of Kim, *et al*. (2018) .

Molecular Identification of Selected *Pseudomonas* Isolate by *16S RNA:*

The isolates was identified by comparing the 16S rRNA gene sequences with the NCBI GenBank database using the Blast program, which the results showed that the *16S RNA* gene was detected in 57/57 (100%) *P. aeruginosa* isolates as in Figure (2), this result is consistent with (Mohamed, *et al*., 2022 ; Noaman *et al*., 2023) , which is an identification gene that determines the identity of bacterial isolates, if they are *P. aeruginosa*. If it does not appear in any isolation, it is excluded and not classified within *P. aeruginosa*.

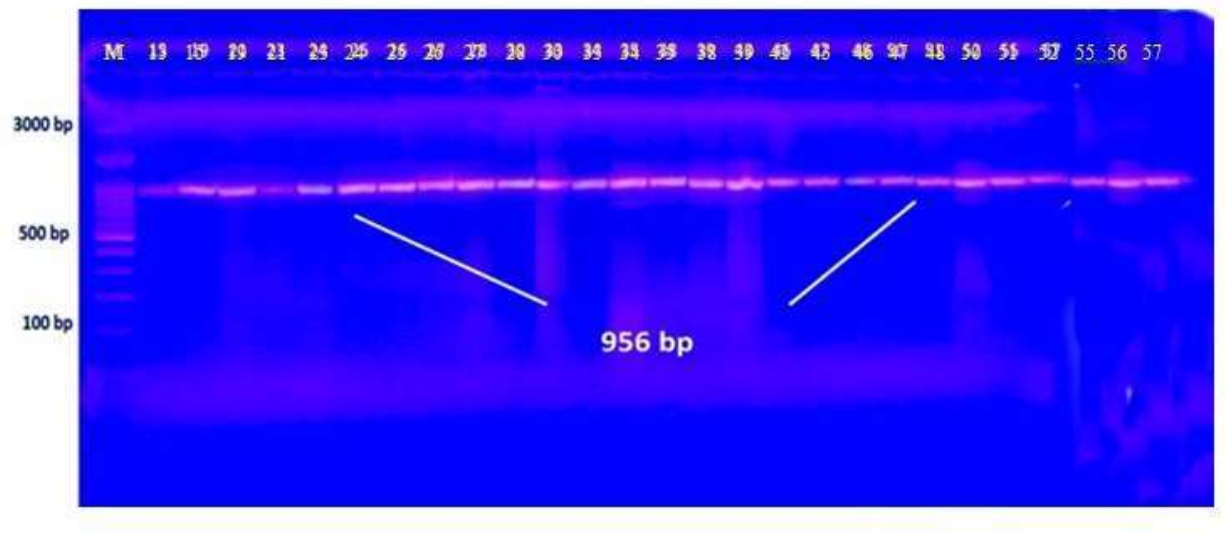

Figure (2): PCR amplification products of *P. aeruginosa* isolates that amplified with *16S RNA* gene primers with product 956 bp. Lane (M), DNA molecular size marker (3000-bp ladder), all samples show positive results with the *16S RNA* gene. Agarose gel electrophoresis (1.5% agarose, 7 V/cm2 for 90min).

The *Las biofilm* gene of *P. aeruginosa* :

The results showed that the *Las biofilm* gene was detected in 43/57 (75.43%) *P. aeruginosa* isolates as in Figure (3), this gene consider as virulence genes with Quorum sensing (QS) to forming the biofilm with other cells .

A crucial function of quorum sensing (QS), an one cell to another density-dependent communication mechanism, is the regulation of virulence factors, antibiotic resistance, and biofilm formation. Three different QS systems, Las, Rhl, and MvfR (PqsR), are present in Pseudomonas aeruginosa. These systems are mediated by small, diffusible signaling molecules known as autoinducers, which include 4-hydroxy-2-alkylquinolines and the N-acyl homoserine lactones N-(3-oxododecanoyl)-l-homoserine lactone (C12-HSL) and N- butanoyl-l-homoserine lactone (C4-HSL). These signaling molecules control virulence factors, bacterial adhesion, and biofilm formation in addition to influencing the synthesis of exoenzymes (Bahari, *et al*., 2017 ; Davarzani, *et al*., 2021).

The samples in this gene did not appear did not form a biofilm or it was very weak, while the samples that contained the *Las* gene had a strong and distinct biofilm formation , this is consistent with what he

mentioned (Miranda, *et al*., 2022; Avinash, *et al*., 2023) .

The negative samples were sourced consecutively (catheter , ear , vaginal , vaginal, ear and UTI) , These samples are all missing the *Las biofilm* gene and are very sensitive to various antibiotics. We can explain their sensitivity because they are unable to form biofilm compared to the rest of the antibiotic-resistant samples that form biofilm because they contain the *Las biofilm* gene, this is fully consistent with the results In terms of burns (Ghasemian, *et al*., 2023) and agree with (Heidari, *et al*., 2022) with results of UTI .

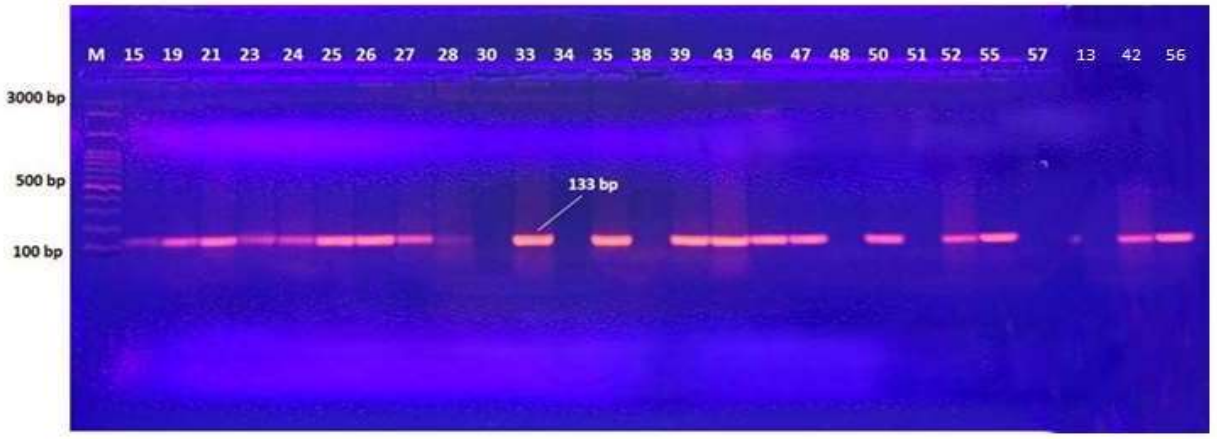

Figure (3): PCR amplification products of *P. aeruginosa* isolates that amplified with *Las biofilm* gene primers with product 133 bp. Lane (M), DNA molecular size marker (3000-bp ladder), 43/57 samples show positive results with the *Las biofilm* gene. Agarose gel electrophoresis (1.5% agarose, 7 V/cm2 for 90min).

The *algD* gene of *P. aeruginosa* :

The biofilms are produced by *P. aeruginosa* in live tissues, on catheters, and in the surroundings, which The exopolysaccharides alginate make up the biofilm structure, which The polymer known as alginate, which comprises α-L-guluronic acid and β-D-mannuronic acid, plays a crucial part in safeguarding and maintaining the structural integrity of biofilms. The majority of the genes that produce alginate are found in a large operon. *AlgD* is the operon's initial gene and is required for the synthesis of alginate, and its expression is fully regulated (Rajabi, *et al*., 2022 ; Al-Mohammed, and Mahmood, 2024).

Results of examination this gene showed that the *algD* gene was detected in 41/57 (71.92%) *P. aeruginosa* isolates as in Figure (4), this virulence genes with synthesis of the biofilm with surfaces and other tissues .

The samples give negative results with *algD* gene were sourced (wound , catheter , catheter, ear, vaginal , vaginal, ear and ear) respectively , these samples were very sensitive to antibiotics and the result of the biofilm formation test was negative for them, while the samples that contained this gene were able to form biofilm in addition to being resistant to antibiotics, as with (Rajabi, *et al*., 2022) also near to (AL-Sheikhly, *et al*., 2020).

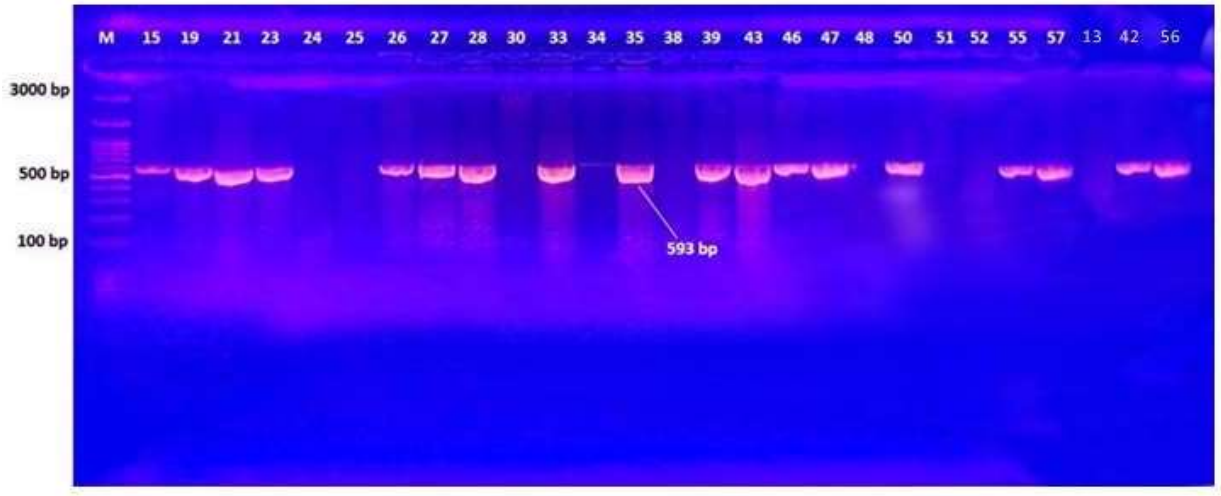

Figure (4): PCR amplification products of *P. aeruginosa* isolates that amplified with *algD* gene primers with product 593 bp. Lane (M), DNA molecular size marker (3000-bp ladder), 41/57 samples show positive results with the *algD* gene. Agarose gel electrophoresis (1.5% agarose, 7 V/cm2 for 90min).

The *oprL* gene of *P. aeruginosa* :

The virulence factor genes (oprI and oprL) produced by *P. aeruginosa* isolated from burn, catheter , wound, ear, urinary tract infections , vaginal and blood were found using a PCR, first, *oprL* was sequenced in order to ascertain the sequence identity of the *P. aeruginosa* gene. Figures (5) show the results of the analysis, which show positive results of samples as 41/57 (71.92%) **,** Adding clarification to that the business Macrogene was able to detect the entire 500 bp, rich in GC (60.2%) *oprL* gene of *P.aeruginosa*. Utilizing the chain termination approach, sequencing was accomplished, the results and analysis are consistent with (Mohammed and Ahmad, 2017 ; Jawher and Hassan 2022).

P. aeruginosa has a number of virulence factors that could increase its pathogenicity, including the outer membrane proteins oprI and oprL, which are crucial to the bacterium's interactions with the environment and to its inherent resistance to antibiotics. These particular outer membrane proteins have also been linked to efflux transport systems that impact cell permeability. Since these proteins are unique to this bacterium, they may be a trustworthy component in the quick identification of *P. aeruginosa* in clinical samples (Mohammed and Ahmad, 2017 ; Ciamak, G. 2022).

This study was characterized by the diversity of sources for samples, so it confirms the results more broadly than studies that dealt with the same topic.

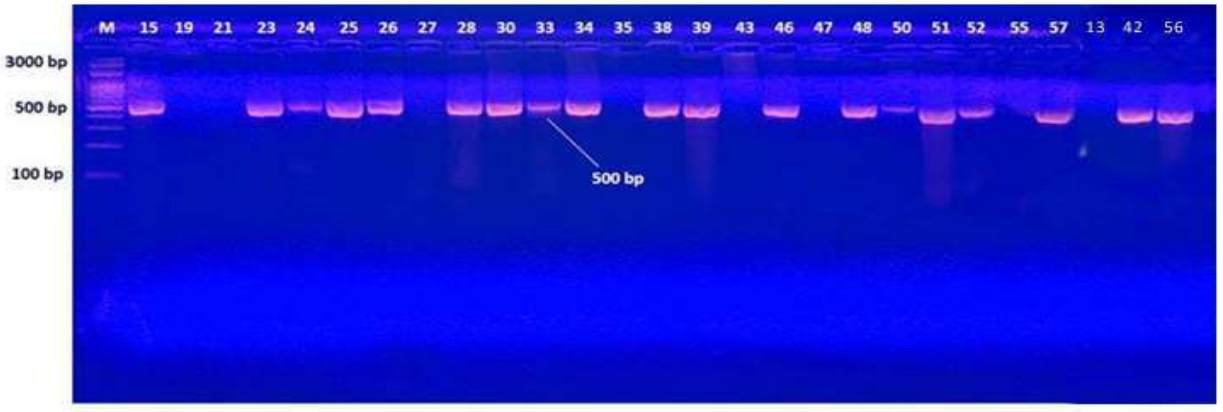

Figure (5): PCR amplification products of *P. aeruginosa* isolates that amplified with *oprL* gene primers with product 500 bp. Lane (M), DNA molecular size marker (3000-bp ladder), Agarose gel electrophoresis (1.5% agarose, 7 V/cm2 for 90min). 41/57 samples show positive results with the *oprL* gene.

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