Original Article

Detection of some virulence factors of Pseudomonas aeruginosa as a result of treatment with antibiotic Levofloxacin and using specialist primer

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Abstract

The current study included a group of (150) clinical swabs for patients suffering from burns, infections in wounds, diabetic foot ulcers and urinary tract infections for both gender, (69) males and (81) females, their ages ranged between 1- 68 years, for the period of November 2021 until the end of February 2022, from Hospitals in Mosul. After the final diagnosis of the samples, (75) isolates of P. aeruginosa were obtained, representing (50%) of the total clinical samples, and the rate of isolation of them from samples of burns, wounds, diabetic foot ulcers and urinary tract infections was 75% (42/56), 41.1% (23/56), 53.3% (8/15) and 8.7% (2/23), respectively.

The antimicrobial susceptibility test was performed by the disk diffusion method, and it was found that the isolates are multi-antibiotic resistant. The percentage of resistance shown by the isolates were 70.7% for Piperacillin , 89.3% for Amikacin, 74.7% for Aztreonam and Levofloxacin , 81.3% for Gentamicin, Ceftazidime and Cefepime, 76% for Ciprofloxacin, 80% for Netilmicin and Tobramycin , and 45.3% for Imipenem.

The effect of the antibiotic Levofloxacin on the PslA gene responsible for the production of biofilms, and the PhzM gene responsible for the production of the pyocyanin pigment by bacteria was studied by extracting the DNA of ten bacterial isolates and then performing a polymerase chain reaction (PCR) using the primers PslA, PhzM, and then performing the electrophoresis process For the final reaction products and to note the presence of Bands before and after the treatment of bacterial isolates with the antibiotic.

Five isolates (PA1, PA4, PA5, PA6, PA7) were selected to perform DNA sequencing before and after treatment with the antibiotic Levofloxacin by sending the PCR products of the five samples with the 16SrRNA primer to the Macrogen biotechnology company to know the sequence of nitrogenous bases using a Genetic analyzer 3130 , Changes were observed in the sequence of nitrogenous bases after treatment with the antibiotic Levofloxacin, four new genes were recorded in the National Center for Biotechnology Information (NCBI), and these genes were named short for the name of the supervisor and researcher.

Keywords: Pseudomonas aeruginosa, Pyocyanin, Biofilm, PhzM, PslA.

1. INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic pathogen . These bacteria can invade the bloodstream, causing bacteremia and then septicemia, and infect immunodeficiency patients such as patients with leukemia, diabetes, Acquired immunodeficiency syndrome (AIDS), severe burn patients, and those who have undergone surgery (1-3).

Pseudomonas aeruginosa is one of the main causes of nosocomial infection, causing (10-15%) of these infections. It is an opportunistic pathogen with the ability to cause various infections such as urinary tract infection, meningitis, bone infection , eye infection, middle ear infections, and respiratory infections, including pneumonia, especially those with cystic fibrosis , and inflammation of the digestive system (4,5).

P. aeruginosa it is Gram negative bacteria that causes diseases due to its possession of many virulence factors that have an effect on its pathogenesis, including flagellum, Pili IV, exoenzyme S that helps it to attach, protease, hemolysine, Phospholipase and elastase (6), toxins such as Exotoxin A and Endotoxin responsible for the fever and shock associated with septicemia, and the production of pigments such as Pyocyanin It is soluble in chloroform and a member of the tricyclic compounds "phenazine" (7).

P. aeruginosa has two homologous operon (phzA1B1C1D1E1F1G1 and phzA2B-2C2D2E2F2G2) to be coded of phenazine-1-

carboxylic acid and two phenazine genes (phzS and phzM) responsible for converting the enzymes of phenazine-1-carboxylic acid to pyocyanin (8). as well as The presence of an alginate layer and a biofilm increases its resistance to antibiotics.

Biofilm formation is another important characteristic of P.aeruginosa contributes to chronic infections as they reduce susceptibility to antibiotics and as a result, treatment options are reduced (9).

Biofilm is defined as an assembly of microbial cells bound (not removed by gentle rinsing) to a surface and covered with an exopolysaccharide matrix (slime) (10).

Bacteria within biofilms can escape host immune responses and resist antimicrobial treatments up to 1,000 times more than their planktonic cells. P. aeruginosa has the ability to produce biofilms, making it an excellent model for studying biofilm formation (11). The flexible biofilm is a critical weapon for bacteria to compete, survive and control in the lung of cystic fibrosis patients (12).

2. MATERIALS AND METHODS:

2.1. Specimen collection:

A total of 150 specimens were collected from patients admitted to the several hospitals in Mosul between November 2021 and March 2022.

2.2. Bacterial identification:

The Vitek-2 automated system by using GramNegative cards according to the manufacturer's instructions was used for diagnosis to the species level . and use 16SrRNA gene by PCR for molecular identification . The isolated bacteria were stored in trypticas soy broth (TSB) with 40% glycerol at -20°C until used.

2.3. Antibiotic Susceptibility Testing:

The susceptibility of isolates to different antibiotics was tested using the Kirby-Bauer disk diffusion method following the Clinical and Laboratory Standards Institute guidelines (13). Using antibacterial agents included: Gentamicin (CN), Tobramycin (Tob), Amikacin (AK), Ciprofloxacin (CIP), Levofloxacin (LEV), Piperacillin (PRL), Ceftazidime (CAZ), Cefepime (FEP), Netilmicin (NET), Aztreonam (ATM), and Imipenem (IMP). On Mueller- Hinton agar plate (Himedia, India) using overnight culture at McFarland standard 0.5 followed by incubation at 35°C for eighteen hours (14).

2.4. Molecular diagnosis of Pseudomonas aeruginosa based on 16srRNA gene.

Bacterial isolates were detected by amplifying the 16srRNA region in the extracted bacteria genome. 4 µl (100 nanogram) of template DNA and 1µl (10 picompl) of each gene-specific primer were added to the contents of the master mix (Table 1).

Table 1: The 16srRNA primer used in the study

Primer	Sequence	Band Size	المصدر
16srRNA Forward	GACCTCGGTTTAGTTCACAGA	1100 bp	(Shaebth , 2019)
16srRNA Revers	CACACGCTGACGCTGACCA		

Then the reaction tubes were inserted into the thermocycler to conduct the multiplication reaction, using the special program for the reaction, as shown in (Table 2):

Table 2: Steps of a polymerase chain reaction (PCR) program

No.	Stage	Temperature	Time	Cycle number
1.	Initial denaturation	95	6 min.	1
2.	denaturation	95	45 sec.	35
3.	Annealing	56	1 min.	
4.	Extension	72	1 min.	
5.	Final extension	72	5 min.	1

2.5. Molecular Detection of Pyocyanin and biofilm Genes.

A number of virulence genes for P. aeruginosa have been molecularly identified by adopting specific programs for each gene with primers designed to verify the presence of the genes within the bacteria, as shown in (Table 3).

Table 3: PslA, PhzM primers used in the study.

Primers	Sequence	tm	Band size	المصدر
PslA F	CACTGGACGTCTACTCCGACGATAT	65	1119	(Nader *et al*., 2017)
PslA R	GTTTCTTGATCTTGTGCAGGGTGTC			
PhzM F	ACGATCATGCGGGTTTCCAT	56	454	(Yuosif *et al*., 2015)
PhzM R	GCGAATTGACCAAGGCCATC			

Also, the reaction tubes were inserted into the thermocycler to conduct the multiplication reaction, using the special program for the reaction, as shown in (Table 2).

2.6. The effect of the antibiotic Levofloxacin on some virulence factors genes.

In this study, 10 isolates of P. aeruginosa were selected to study the effect of the antibiotic Levofloxacin on the genes of some virulence factors represented by the gene responsible for the formation of biofilms (PslA) and the gene responsible for the production of the pyocyanin pigment (PhzM).

Where the DNA of the ten bacterial isolates was extracted and gel electrophoresis was carried out for the isolates to ensure the purity of the extracted DNA.

Polymerase chain reaction (PCR) was performed to increase the gene to be detected as described in paragraph (2.5) , and to use specialized primers (PslA and PhzM) responsible for the formation of biofilms, and the production of pyocyanin pigment, respectively (table 3) .

Gel electrophoresis of the results of PCR reactions and gel imaging was carried out using UV Trans illumination.

After that, the ten bacterial isolates were treated with the antibiotic Levofloxacin, and the lower concentration of the minimum inhibitory concentration (Sub-MIC) was selected. The DNA was extracted from the ten bacterial isolates after treatment with the antibiotic.

Also, the same previous steps of PCR reactions and electrophoresis of the results of PCR reactions were performed for bacterial isolates after treatment with the antibiotic Levofloxacin.

2.7. Determination of nucleotide sequencing of amplified pieces using DNA sequencing technology and recording of new genes.

The sequence of the nitrogen bases of the bacterial samples under study was determined. All the final PCR products of the samples with the primers were sent to Bioneer (Korea) and the sequence was read for the genes based on the 3130 Genetic Analyzer device supplied by the Japanese company Hitachi.

The gene-specific sequences were matched to the gene sequences documented in the National Center Biotechnology Information NCBI by uploading the sequences obtained on the GenBank BLAST website (https://www.ncbi.nlm.nih.gov) for comparison with other global sequencing to identify types of bacterial isolates.

The bacterial sequence of isolates (PA1, PA4, PA5, PA6) was sent again after treatment with Levofloxacin at Sub-MIC to GenBank to record the isolates bacteria in its data.

3. RESULTS AND DISCUSSION

3.1. Bacterial Isolates.

A total of 150 specimens were included in the study. P. aeruginosa was present in 75 of various clinical specimens, representing (50%) of the total clinical samples, and the rate of isolation of them from samples of burns, wounds, diabetic foot ulcers and urinary tract infections was 75% (42/56), 41.1% (23/56), 53.3% (8/15) and 8.7% (2/23), respectively.

The incidence of P. aeruginosa infection was higher in men than in females, 42 (56%) and 33 (44%) respectively. The results of the current study agree with the study of (15) of bacteria isolated from burn patients, which showed that the percentage of males was (77%) and females (23%), and also agrees with the study (Yolbaş et al., 2013), where This study showed infection rate for males (70%) and females (30%).

3.2. Antimicrobial Susceptibility of P. aeruginosa isolates.

The results of the current study showed that all P. aeruginosa isolates had a difference in the resistance to the antibiotics used in this study , Figure (1). It was noted from the results of the current study that the highest antibiotic resistance was to the antibiotics Amikacin, Gentamicin, Ceftazidime, Cefepime, Netilmicin, Tobramycin; 89.3%, 81.3%, 81.3%, 81.3%, 80%, 80% respectively.

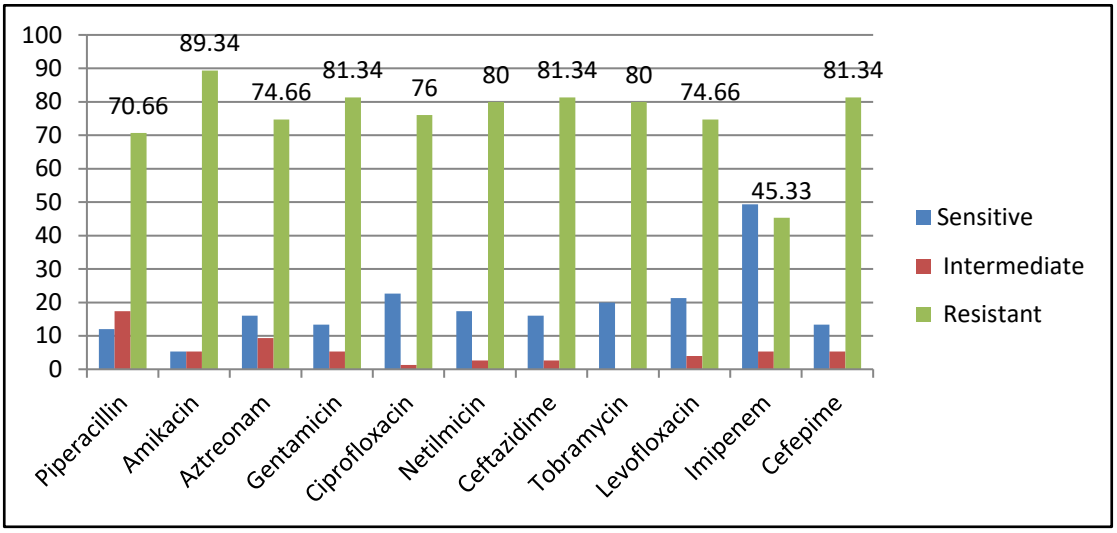

Fig. 1: Percentage of resistance of P. aeruginosa isolates to antibiotics.

The results of the current study were consistent with the results of (17). Whereas, P. aeruginosa isolates showed high resistance to Ceftazidime (81%), Cefotaxime (78%), Piperacillin (76%) and were resistant to Ciprofloxacin, Tobramycin (74%) and Genamicin (72%). Amikacin and Meropenem (70%) , Ofloxacin (66%) and Imipenem resistant (65%).

Wounds and burns of hospital patients can be contaminated with P. aeruginosa , due to the presence of this antibiotic-resistant bacterium in the hospital environment, and it can also be found in tap water, latrines, as well as hospital care workers; Thus, bacteria are transmitted from one patient to another. The cause of antibiotic resistance may be attributed to the concerted action of multi-drug efflux pumps with chromosomally encoded antibiotic resistance genes (eg, mexXY) and reduced permeability of bacterial cell membranes. Besides intrinsic resistance, P. aeruginosa develops easily acquired resistance either by chromosomal mutations in chromosomal-encoded genes, or by the process of gene transfer for determinants of antibiotic resistance. Resistance to antibiotic aminoglycosides is caused by P. aeruginosa's production of modified enzymes such as phosphotransferase and N-acetyl-transferase, the genes for these enzymes being transferred on a plasmid or chromosome (18).

3.3. Molecular diagnosis of Pseudomonas aeruginosa based on 16srRNA gene.

The results of PCR amplification reactions using the Thermocycler showed the efficiency of the extracted DNA in molecular biological experiments and tests. The bacterial isolates to which the extracted DNA samples belonged were diagnosed by using 16srRNA special primers for diagnosis , Figure (2).

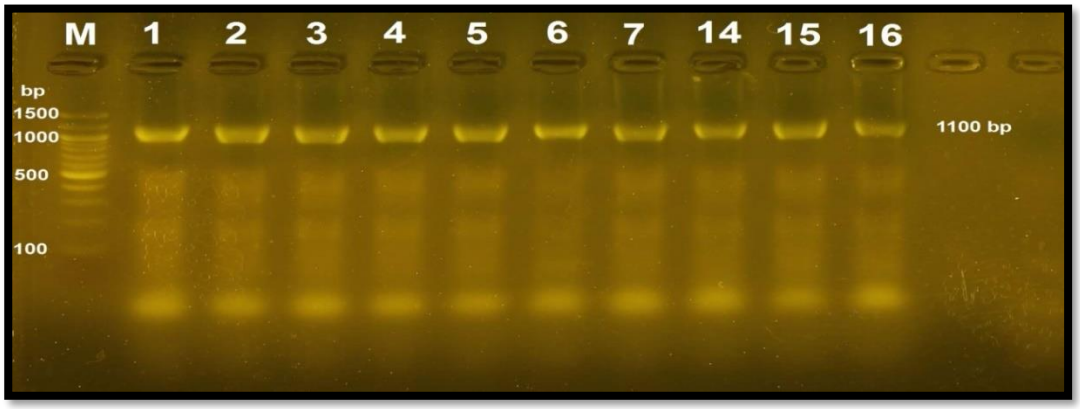

Fig. 2: The PCR product of P. aeruginosa samples shows the 16SrRNA region and the bp1100 reaction product, where M is Ladder.

3.4. The effect of the antibiotic Levofloxacin on some virulence factors genes.

Ten bacterial isolates of P. aeruginosa were selected to study the effect of the antibiotic Levofloxacin on the biofilm genes using the primer (PslA) and on the genes for the production of the pyocyanin pigment using the primer (PhzM) previously mentioned in table 3.

The genes responsible for the formation of biofilms and the genes responsible for the production of pyocyanin pigment were investigated for bacterial isolates before treatment with the antibiotic Levofloxacin (Figure 3).

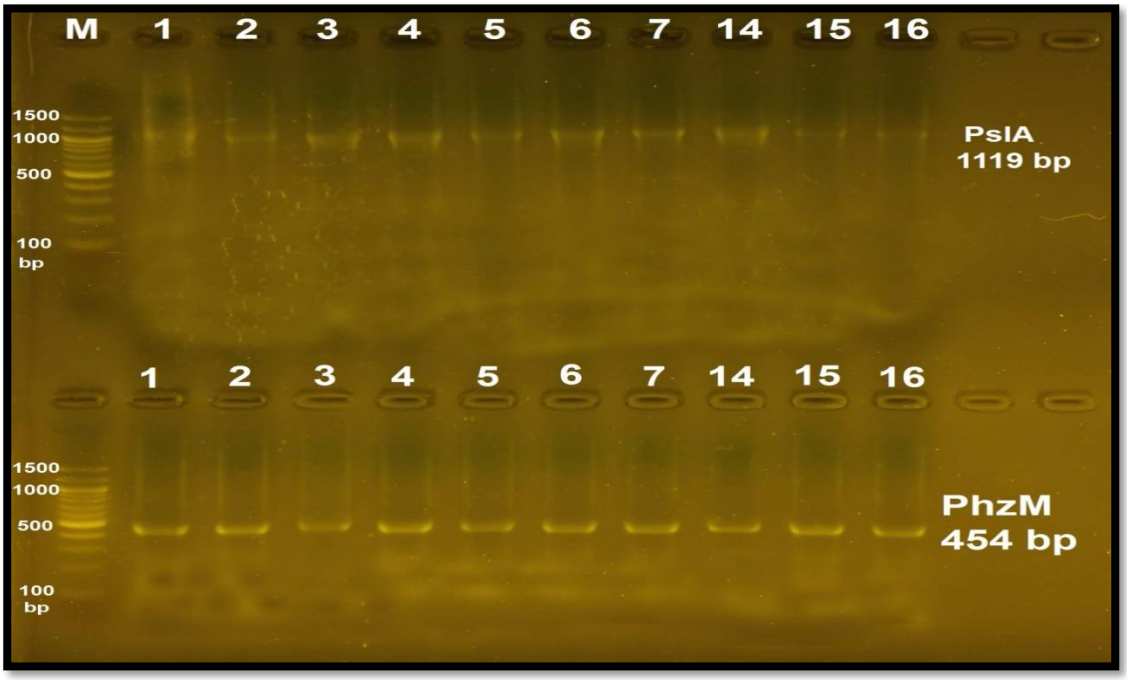

Fig. 3: Electrophoresis of PCR products using the primers PslA 1119 bp and PhzM 454bp, before treatment with Levofloxacin, where M is the ladder.

Then the same bacterial isolates were treated with the antibiotic Levofloxacin and the concentration of the antibiotic less than the minimum inhibitory concentration (Sub-MIC) was selected, Figure 4.

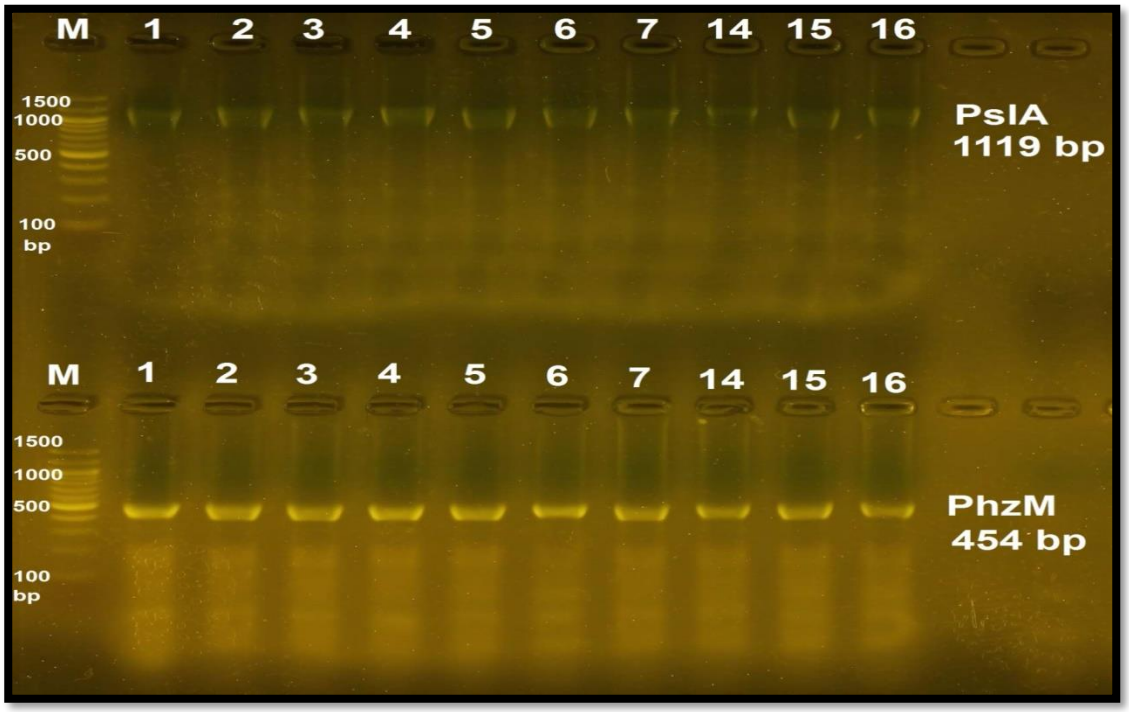

Fig. 4: Electrophoresis of PCR products using the primers PslA 1119 bp and PhzM 454bp, after treatment with the antibiotic Levofloxacin, where M is the ladder.

By conducting the process of electrophoresis of the PCR products of the DNA extracted from bacterial samples after treatment with the antibiotic, it is found that there is no effect on the genes investigated using the PslA and PhzM primers, as the treatment of bacteria with the antibiotic does not cause the gene to disappear completely, but the gene remains present However, the gene expression decreases, as is evident when quantitatively measuring the production of biofilms using the microtiter plate (19) , and quantitatively measuring the production of pyocyanin pigment (20), where it is found that the antibiotic Levofloxacin has an effect Inhibitory works to reduce the production of biofilm and reduce the production of Pyocyanin pigment.

3.5. Determination of nucleotide sequences for amplified pieces using DNA sequencing

After DNA extraction from P. aerugenosa isolates, five bacterial isolates (PA1, PA4, PA5, PA6, PA7) were selected to perform DNA sequencing before and after treatment with the antibiotic Levofloxacin by sending the PCR products of the five samples with the 16SrRNA Primer to Macrogen biotechnology company to determine the sequence of nitrogen bases using a Genetic analyzer 3130.

The results of the nitrogen base sequences of the gene for isolates (PA1, PA4, PA5, PA6, PA7) befor treatment with levofloxacin were compared within the National Center for Biotechnology Information (NCBI) using the BLAST program , the Sequence ID is (MW647093.1 , KF613156.1 , KT819276.1 , MN640840.1 , FM173819.1) respectively.

Through the results, techniques based on DNA amplification can detect the amounts of bacterial DNA, as the examination with them is usually sensitive and rapid, and thus can overcome the limitations of traditional diagnostics that are slow or have insufficient sensitivity (21). There may be errors in bacterial diagnosis using traditional methods, while molecular diagnostic methods compare the sequence of nitrogenous bases of unknown bacterial genera with known bacterial genera within a database based on amplification of a region within the DNA. Because of the accuracy of this method, it can diagnose different types of bacteria within the genus. The one as well as the strains within the species that are difficult to distinguish based on the external appearance of the bacterial colonies.

3.6. The effect of the antibiotic Levofloxacin on the 16SrRNA gene and the registration of new genes within Pseudomonas aeroginosa bacteria.

The PCR reaction products of the five samples after treatment with the antibiotic Levofloxacin at Sub-MIC concentration with the 16SrRNA primer were sent to the Macrogen biotechnology company to determine the sequence of nitrogen bases using a Genetic Analyzer 3130, and new genes were discovered that were registered globally in the National Center for Biotechnology Information (NCBI).

Where 4 new isolates were recorded in the name of both the supervisor and the researcher, as the international number of the bacteria gene was LC705276.1, and the gene was named Ah-Q1 gene after treating the isolate (PA1) with an antibiotic.

The international number of the bacteria gene is LC705277.1, and the gene was named Ah-Q2 gene after isolate (PA4) was treated with an antibiotic.

The international number of the bacteria gene is LC705278.1 and the gene was named Ah-Q3 gene after treating the isolate (PA5) with an antibiotic.

The international number of the bacteria gene is LC705279.1 and the gene was named Ah-Q4 gene after treating the isolate (PA6) with an antibiotic. The new genes were named Ah-Q, short for the supervisor and researcher (Table 4).

As for the isolate (PA7), after the changes that occurred in the arrangement of its nitrogenous bases as a result of its treatment with the antibiotic, it became the isolate with the international number LN589738.1.

Table (4): The four bacterial isolates before and after treatment with the antibiotic Levofloxacin at Sub-MIC concentration. New genes were recorded at the NCBI site.

Isolate name after treatment	GenBank after treatment	Isolate name befor treatment	GenBank befor treatment	Source of isolation	Isolate No.
Pseudomonas aeruginosa Ah-Q1 gene for 16S rRNA	LC705276.1	***Pseudomonas fluorescens* strain Pf2010 16S ribosomal RNA gene**	MW647093.1	Burn patients	**PA1**
Pseudomonas aeruginosa Ah-Q2 gene for 16S rRNA	LC705277.1	*Pseudomonas sp.* PS-H 16S ribosomal RNA gene	KF613156.1	Burn patients	**PA4**
Pseudomonas aeruginosa Ah-Q3 gene for 16S rRNA	LC705278.1	***Pseudomonas aeruginosa* strain A361 16S ribosomal RNA gene**	KT819276.1	Burn patients	**PA5**
Pseudomonas aeruginosa Ah-Q4 gene for 16S rRNA	LC705279.1	***Pseudomonas aeruginosa* strain BGR1I1 16S ribosomal RNA gene**	MN640840.1	Burn patients	**PA6**

On the National Center for Biotechnology Information (NCBI) site, after typing the new GenBank isolate number in the search field, the following information about the new isolate appears.

The first isolate Ah-Q1 gene with the number LC705276.1:

Pseudomonas aeruginosa Ah-Q1 gene for 16S rRNA, partial sequence.

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2022

DEFINITION  Pseudomonas aeruginosa Ah-Q1 gene for 16S rRNA, partial sequence.

ACCESSION   LC705276
VERSION     LC705276.1
KEYWORDS    .
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SOURCE Pseudomonas aeruginosa
ORGANISM Pseudomonas aeruginosa
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.

REFERENCE 1
AUTHORS Zaki,A.M.
TITLE determining the emergence and disappearance of some virulence factors of Pseudomonas aeruginosa bacteria as a results of treatment with some antibiotics and using specialized primer

JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1045)
AUTHORS Zaki,A.M. and Attia,Q.M.
TITLE Direct Submission
JOURNAL Submitted (09-APR-2022) Contact:Ahmed Mazin Zaki University of Tikrit, College of Science, Department of Biology; Alzohoor, Mosul, Ninava 09334, Iraq

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The Second isolate Ah-Q2 gene with the number LC705277.1:

Pseudomonas aeruginosa Ah-Q2 gene for 16S rRNA, partial sequence.

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2022
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Pseudomonadaceae; Pseudomonas.

REFERENCE   1
  AUTHORS   Zaki,A.M.
  TITLE     determining the emergence and disappearance of some virulence
factors of Pseudomonas aeruginosa bacteria as a results of treatment with some
antibiotics and using specialized primer

JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 977)
  AUTHORS   Zaki,A.M. and Attia,Q.M.
  TITLE     Direct Submission

JOURNAL     Submitted (09-APR-2022) Contact:Ahmed Mazin Zaki University of
Tikrit, College of Science, Department of Biology; Alzohoor, Mosul, Ninava
09334, Iraq

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The Third isolate Ah-Q3 gene with the number LC705278.1:

Pseudomonas aeruginosa Ah-Q3 gene for 16S rRNA, partial sequence.

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2022

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  ORGANISM  Pseudomonas aeruginosa
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; Pseudomonas.

REFERENCE   1
  AUTHORS   Zaki,A.M.
  TITLE     determining the emergence and disappearance of some virulence
factors of Pseudomonas aeruginosa bacteria as a results of treatment with some
antibiotics and using specialized primer

  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 397)
  AUTHORS   Zaki,A.M. and Attia,Q.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-APR-2022) Contact:Ahmed Mazin Zaki University of
Tikrit, College of Science, Department of Biology; Alzohoor, Mosul, Ninava
09334, Iraq
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The fourth isolate Ah-Q4 gene with the number LC705279.1:

Pseudomonas aeruginosa Ah-Q4 gene for 16S rRNA, partial sequence.

LOCUS LC705279 432 bp DNA linear BCT 13-APR-2022

DEFINITION Pseudomonas aeruginosa Ah-Q4 gene for 16S rRNA, partial sequence.
ACCESSION LC705279
VERSION LC705279.1
KEYWORDS .
SOURCE Pseudomonas aeruginosa
ORGANISM Pseudomonas aeruginosa
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas.

REFERENCE 1
AUTHORS Zaki,A.M.

TITLE determining the emergence and disappearance some virulence factors of Pseudomonas aeruginosa bacteria as a results of treatment with some antibiotics and using specialized primer.

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REFERENCE 2 (bases 1 to 432)
AUTHORS Zaki,A.M. and Attia,Q.M.
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JOURNAL Submitted (09-APR-2022) Contact:Ahmed Mazin Zaki University of Tikrit, College of Science, Department of Biology; Alzohoor, Mosul, Ninava 09334, Iraq

FEATURES Location/Qualifiers
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3.7. The Phylogeny among bacterial isolates.

The MEGAX program was used to analyze and interpret the results of the polymerase chain reaction (PCR) to estimate the differences in DNA among five isolates of P.aeruginosa bacteria (PA1, PA4, PA5, PA6, PA7) before and after treating the five bacterial isolates with the antibiotic Levofloxacin at the concentration Sub-MIC of each isolate , Figure (5) showed the extent of genetic affinity and divergence between bacterial isolates , and shows the extent of genetic affinity between isolate LC705278.1 (PA5 After) and isolate LC705276.1 (PA1 After), as they are two isolates that were registered in the NCBI International Information Bank in the name of both the supervisor and the researcher, and that isolate LC705278.1 (PA5 After) is genetically divergent with isolate (PA5 Before), and isolate LC705276.1 (PA1 After) is also genetically divergent with isolate (PA1 Before) after it was treated with the antibiotic Levofloxacin at Sub-MIC concentration.

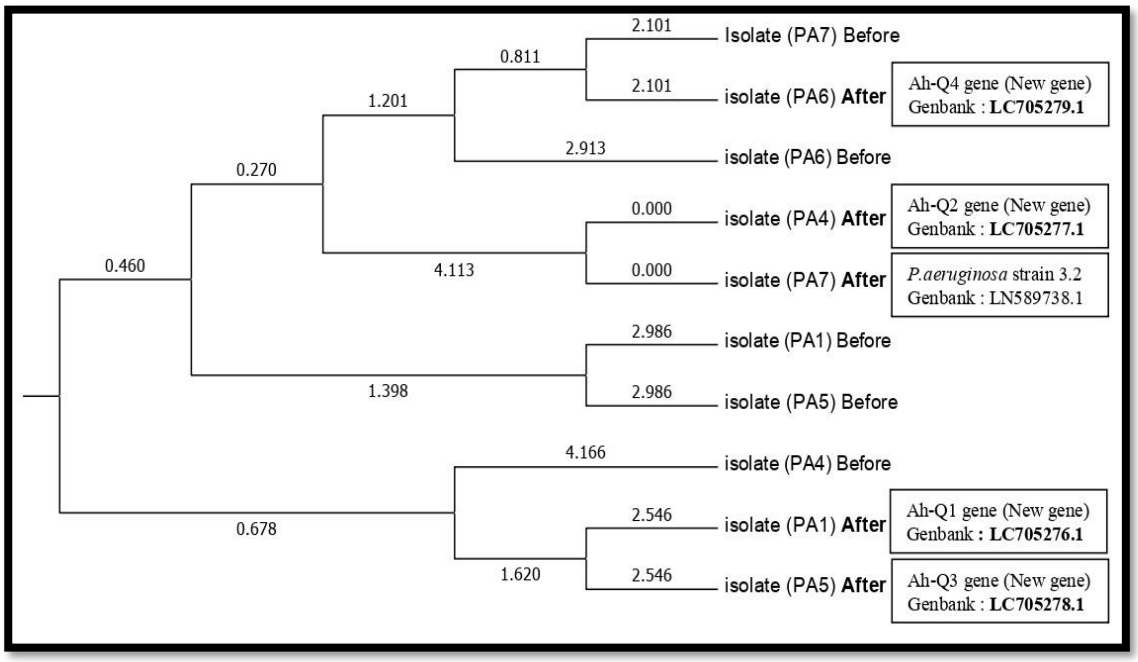

Fig. 5: Scheme showing the genetic relationship of five P.aeruginosa isolates before and after treatment with Levofloxacin at Sub-MIC using MEGAX software.

Also, isolate LC705277.1 (PA4 After) and isolate LN589738.1 (PA7 After) are genetically closely related. Isolate (PA7 Before) and isolate LC705279.1 (PA6 After) also show a genetic affinity between them.The diagram shows that all five bacterial isolates (PA1, PA4, PA5, PA6, PA7) before being treated with the antibiotic Levofloxacin had genetic divergence with the isolates (LC705276.1, LC705277.1, LC705278.1, LC705279.1 and LN589738.1) respectively after treatment with Levofloxacin at Sub-MIC concentration.

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