Study the sequences of *exoU* and *toxA* genes in *Pseudomonas aeruginosa*

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Abstract

Background: *Pseudomonas aeruginosa*, a human opportunistic Gram-negative pathogen, is one of the most important nosocomial pathogens and is a major health problem, primarily in immunocompromised individuals.

Aims: To detect *exoU* and *toxA* genes in *Pseudomonas aeruginosa* isolates with PCR techniques and study their sequencing. Materials and Methods: 210 swabs were collected from patients suffering burns and wound infections in both genders, for the period from November 2019 to the end of February 2020.

Results: After final diagnosis of samples, 50(23.80%) isolates of *P. aeruginosa* were obtained. The PCR was carried out for all bacterial isolates, using specialized primers, to detect these two genes (*toxA* and *exoU*). The results showed that the twenty *P. aeruginosa* isolates have the *toxA* gene in a percentage 40% from all isolates. The results also showed that the same 20 *P. aeruginosa* isolates have the gene *exoU* in a percentage 40% from all isolates. The *toxA* and *exoU* genes were amplified by PCR method; the sequencing result of *exoU* gene showd that *P. aeruginosa* having Two Transition G>A code GGC\AAC of amino acid Glycine > Asparagine and Predicted effect Missense, also one Transversion T >A code

GAA>CAA amino acid change Leucine to Histidine the effect Missense. From the Gene Bank, found that part of *exoU* gene having 99% compatibility with subject of *exoU* gene. While the analysis of the *exoU* gene was coordinated by 99% having two Transition of G>A, and C>T code GGC>GAT and amino acid transformation Glycine\ Aspartic acid the effect Missense. The of sequencing for *toxA* gene shows Compatibility of 100% in Gene Bank of *toxA* gene, so no recorded change noticed.

Conclusion: The *P. aeruginosa* which isolated from wounds and burns possessed virulence genes, *toxA* and *exoU* genes. The *exoU* and *toxA* genes having Transition, amino acid alteration and amino acid transformation.

Keywords: *Pseudomonas aeruginosa, exoU, toxA, burn, wound infection.*

دراسة التسلسل الجيني لجيني exoU و toxA في بكتريا الزائف الزنجارية

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الخلاصة

الخلفية: *Pseudomonas aeruginosa* ، أحد مسببات الأمراض الانتهازية البشرية سالبة الكرام، وهي واحدة من أهم مسببات الأمراض في المستشفيات وهي مشكلة صحية كبيرة، خاصة في الأفراد الذين يعانون من نقص المناعة. الأهداف: لاكتشاف جينات *exoU* و *toxA* في عزلات *Pseudomonas aeruginosa* بتقنيات تفاعل البوليميراز المتسلسل ودراسة تسلسلها. المواد وطرائق العمل: تم جمع 210 مسحة لمرضى يعانون من حروق وإلتهابات جروح من كلا الجنسين للفترة من تشرين الثاني 2019 وحتى نهاية شباط 2020. النتائج: بعد التشخيص النهائي للعينات تم الحصول على 50 عزلة (23.80٪) من *P. aeruginosa*. تم إجراء اختبار تفاعل البوليميراز المتسلسل (PCR) لجميع العزلات البكتيرية باستخدام بادئات متخصصة للكشف عن هذين الجينين (ذيفان أ و إكسو). أوضحت النتائج أن عشرين عزلة من *P. aeruginosa* تحتوي على جين *toxA* بنسبة 40٪ من جميع العزلات. كما أظهرت النتائج أن نفس 20 عزلة *P. aeruginosa* لها جين *exoU* بنسبة 40٪ من جميع العزلات. تم تضخيم جينات السموم والجينات الخارجية بطريقة تفاعل البوليميراز المتسلسل. أظهرت نتيجة التسلسل لجين *exoU* أن *P. aeruginosa* لها انتقالان G> رمز AAC \ GGC للحمض الأميني Glycine> Asparagine والتأثير المتوقع Missense، وأيضًا تحويل GAA> CAA A code T> يغير الحمض الأميني

Leucine إلى Histidine التأثير Missense. من بنك الجينات، وجد أن جزءًا من جين *exoU* متوافق بنسبة 99 ٪ مع موضوع جين *exoU*. في حين تم تنسيق تحليل الجين *exoU* بنسبة 99٪ بوجود انتقاليين لـ A > ، و C> T code GGC> GAT وتحويل الأحماض الأمينية lycine / spartic acid، أثر Missense. يُظهر تسلسل جين *toxA* توافقًا بنسبة 100٪ في بنك الجينات لجين ذيفانوكسا، لذلك لم يلاحظ أي تغيير مسجل. الخلاصة: إن فصيلة *P. aeruginosa* المعزولة من الجروح والحروق تمتلك جينات ضراوة وجينات *toxA* و *exoU*. جينات *exoU* و *toxA* لها انتقال وتغيير الأحماض الأمينية وتحول الأحماض الأمينية.

الكلمات المفتاحية : *الزائفة الزنجارية ، exoU toxA الحرق ، اصابة الجروح .*

Introduction

Pseudomonas aeruginosa, is a Gram-negative bacterium that is one of the most common nosocomial pathogens and a major public health concern, particularly in immunocompromised people. It causes infections in a variety of tissues, including the respiratory, urinary, and gastrointestinal tracts, as well as skin infections **[**1**]**. It's the leading source of ventilator-associated pneumonia and burn wound infections, all of which have a high mortality rate of more than 30% **[**2**]**. Due to the breakdown of skin as the first line of innate immunity, this pathogen is the leading cause of infections in burn patients. Furthermore, it accounts for about 77% of burn case deaths over the last 25 years **[**3**]**. The virulence factor is a factor that determines how dangerous something is. Type II secretion mechanisms, which use a pilus-like apparatus to secrete proteins such as lipase, phospholipase, alkaline phosphatase, and protease into the extracellular environment, secrete exotoxin A. Furthermore, exotoxin A has been implicated in local tissue injury and invasion **[**4**]**. So, the objectives of the study are: Isolation of *P. aeruginosa* from clinical samples (Burns, wounds swabs); Diagnosis of *P. aeruginosa* using culture media, and Biochemical methods; then detection of the *exoU* and *toxA* genes in *P. aeruginosa* isolates with Real time PCR techniques and study their sequences.

Methods

A. **Samples collection:** The current study included a collection of (210) swabs from patients suffering from burns and wound infections in both genders, (93) males and (117) females, whose ages ranged from 5-78 years, for the period from November 2019 to the end of February 2020, from Burns departments at Teaching Medical City Hospital; Martyr Ghazi Al-Hariri Hospital for Specialized surgery; Baghdad Teaching Hospital; Burns Hospital Specialized; Educational laboratories\ Medical City Hospital; Al-Karama Teaching Hospital and Al-Yarmouk Teaching Hospital-Baghdad / Iraq.

B. **Identification of bacterial isolates:** *Pseudomonas aeruginosa* isolates were identified and confirmed by utilizing conventional microbiological methods. The samples were cultivated on Blood agar, MacConkey agar and Cetrimide agar to study the phenotypes of P. aeruginosa colonies. King A medium (Oxoid, England) and King B medium (Oxoid, England) were prepared as described in [5] to detect P. aeruginosa. As well as, The Gram stain was used for identification of P. aeruginosa in the samples [6]. Catalase test, Oxidase test, IMVIC test as described by [7]. Motility test was done by using motility medium [7]. The bacterial growth test at 42°C was done by using nutrient agar plates, the positive result is it growth at this temperature [7].

C. **DNA Extraction:** The bacterial DNA extracted from the isolates of P. aeruginosa which grown in culture media, according to the protocol of the supplying company (ZR Fungal/ Bacterial/ Yeast DNA MiniPrepTM D6005). The primer solutions were prepared according to the manufacturer's instructions (Integrated DNA Technologies company; IDT, Canada) by dissolving them in deionized distilled water (ddH2O) to obtain a final concentration of 100 pmol\µl as stock solution. Stock solutions of

the primers were kept at -20 °C until use. To prepare 10 pmol/µl concentration as work primer suspended; 10 µl of stock solution was taken and added to 90 µl of ddH2O, then it mixed with a vortex device and kept at a temperature of -20 °C until use. The toxA primer pairs forward 5'-GGTAACCAGCTCAGCCACAT-3', toxA reverse 5'-TGA TGT CCA GGT CAT GCT TC -3' [8]. The primer sequences for exoU gene used in this study, exoU forward 5'-ACC AGC TCA GCC ACA TGT C -3', exoU reverse 5'-CGC TGG CCC ATT CGC TCC AGC GCT-3' [9].

D. PCR for genes detection: The concentration and purity of extracted bacterial DNA of P. aeruginosa isolates were determined according to Nakayama et al., 2016. Then, the reaction mixture was prepared using a Maxime PCR pre-Mix kit (i-Taq) 20l according to the manufacturer's instructions (iNtRON). The optimum conditions of exoU and toxA gene detection were (initial denaturation 95 ^{o}C, 5 min., 1 cycle; denaturation 95 ^{o}C, 45sec., 35 cycle; annealing 60 ^{o}C, 45sec., 35 cycle; extension 72 ^{o}C, 45sec., 35 cycle; final extension 72^{o}C, 7 min., 1 cycle).

E. Detection of amplified DNA bands: the presence of the amplified DNA bands was investigated [10, 11].

F. Sequencing and Sequence Alignment: Gel Extraction (Sequencing) protocol was done according to (12). The polymerase chain reaction (PCR) products were separated on 2% agarose gel, and imaged by exposure to ultraviolet light at a wavelength of 302 nm after staining with Red safe. Genetic sequencing was performed by the National Hardware Center (microgene - Korea), Biotechnology Laboratory, using the DNA Sequencer 3730XL device. Homology research was performed using the Basic Local Alignment Search Tool (BLAST) software available at the National Center for Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) and the BioEdit program.

G. Statistical Analysis: Descriptive statistics used for categorical data were presented in the form of frequency and percentages. Chi-square test used to find-out the association between categorical data. P-values less than 0.05 were considered statistically significant throughout this study.

Results and discussion

(50) isolates of *P. aeruginosa* were obtained (23.8%). *P. aeruginosa* bacteria are the most often detected bacteria from burn injuries and wounds in higher numbers, according to several surveys. *P. aeruginosa* bacteria were isolated from 52.5% of all swabs from patients in Baghdad hospitals **[**13**]**. The present results were agreed with other researchers in Saudi Arabia **[**14**]**, as they found that 31.6% of swabs had *P. aeruginosa*.

The morphology of bacterial colonies on selective culture medium was used to diagnose *P. aeruginosa*, which was consistent with previous research findings **[**15**]**. The isolates gave positive results for growth at 42 °C and on Cetrimide agar; catalase; oxidase; Voges- Proskauer and motility.

The concentrations of all 50 DNA bacterial isolates were between 54 – 337.5 ng/µL; and the DNA purity ranges from 0.76 - 2. The results showed that the 20 *P. aeruginosa* isolates (8 Burn, 12 wound) under study have the *toxA* gene in a percentage 40% from all isolates. The resulting bands have a molecular weight (352 bp) (fig. 1 A and B).

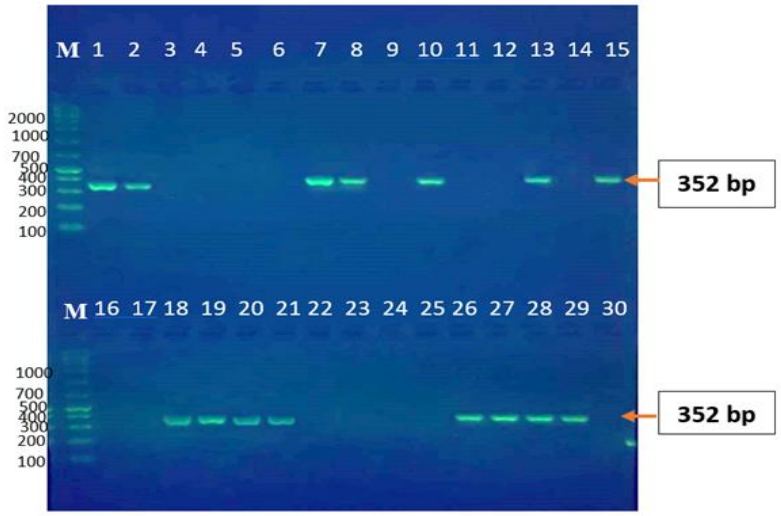

Figure (1-A): Electrophoresis of the polymerase chain reaction products for *toxA* gene, the band size is 352 bp. The product was electrophoresed on 2% agarose at 70 volt/cm^2 for 60min. M: DNA ladder Marker (100bp). The lines 1, 2, 7, 8, 10, 13, 15,18, 19,20, 21, 26, 27, 28, 29 are the *P. aeruginosa* isolates having *toxA* gene.

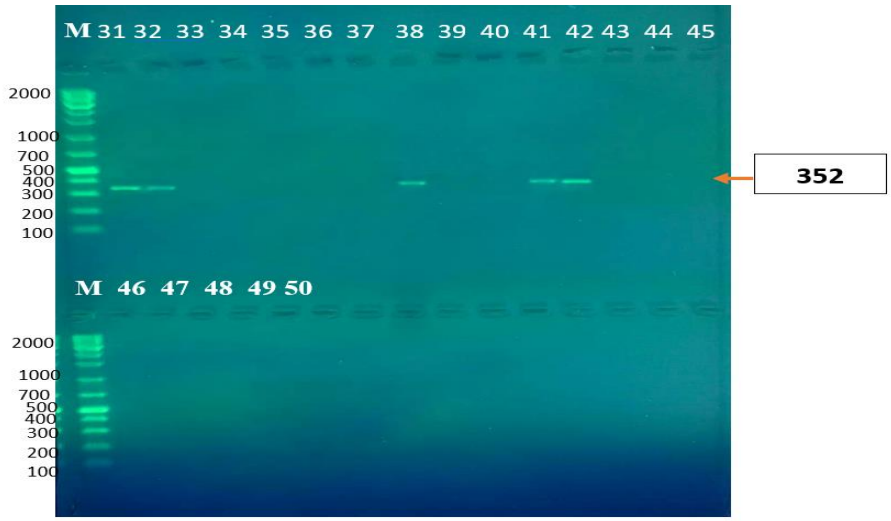

Figure (2-B): Electrophoresis of the polymerase chain reaction products for *toxA* gene, the band size is 352 bp. The product was electrophoresed on 2% agarose at 70 volt/cm^2 for 60min. M: DNA ladder Marker (100bp). The lines 31, 32, 38, 41, 42 are the *P. aeruginosa* isolates having *toxA* gene.

The results also showed that the same 20 *P. aeruginosa* isolates have the *exoU* gene in a percentage 40% from all isolates which have a molecular weight (400 bp), as shown in the figures 2 A and B.

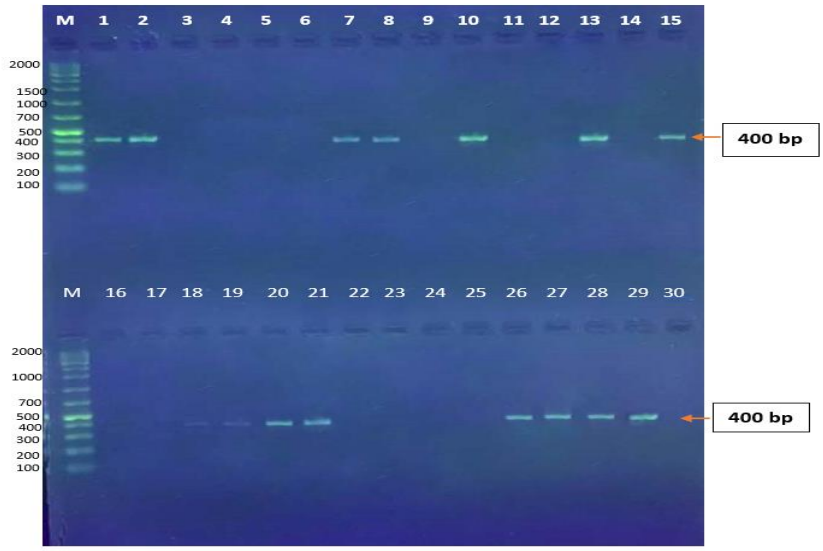

Figure (2-A): Electrophoresis of the polymerase chain reaction products for *exoU* gene, the band size is 400 bp. The product was electrophoresed on 2% agarose at 70 volt/cm^2 for 60min. M: DNA ladder Marker (100bp). The lines 1, 2, 7, 8, 10, 13, 15, 18, 19,20,21, 26,27, 28, 29 are having the *P. aeruginosa* isolates having *exoU* gene.

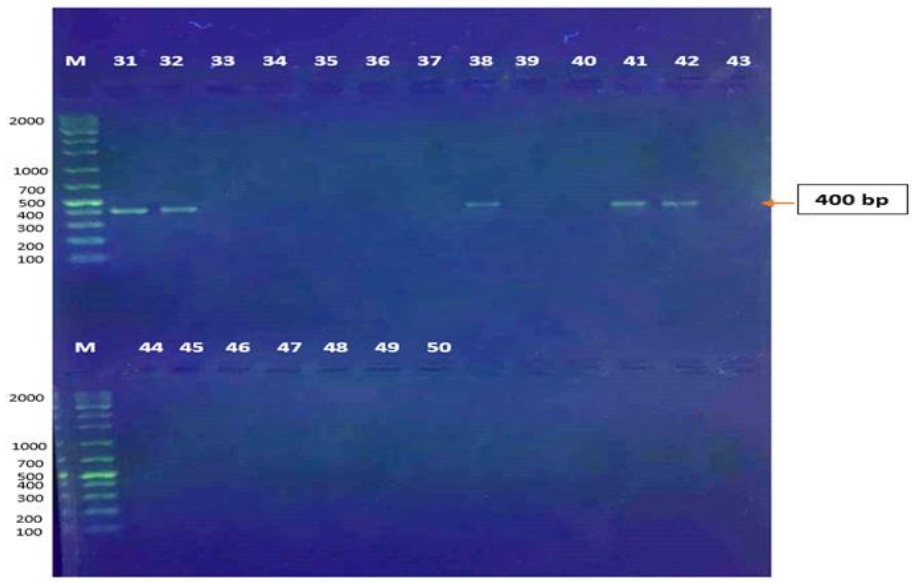

Figure (2-B): Electrophoresis of the polymerase chain reaction products for *exoU* gene, the band size is 400 bp. The product was electrophoresed on 2% agarose at 70 volt/cm^2 for 60min. M: DNA ladder Marker (100bp). The lines 31, 32, 38, 41, 42 are the *P. aeruginosa* isolates having are the *P. aeruginosa* isolates having *exoU* gene.

The toxA and exoU genes were amplified by PCR method, and sent for sequencing service to Macrogen company Korea. The sequencing result of exoU gene shows for *P. aeruginosa* having Two Transition G>A code GGC\AAC of amino acid Glycine > Asparagine and Predicted effect Missense, also one Transversion T>A code GAA>CAA amino acid change Leucine to Histidine the effect Missense. From the Gene Bank, found that part of *exoU* gene having 99% compatibility with subject of exoU gene in NCBI under Sequence ID: MH373640.1, as seen in Figure (3). While the analysis of the *exoU* gene for *P. aeruginosa* in the current study was coordinated by 99% having two Transition of G>A, and C>T code GGC>GAT and amino acid transformation Glycine\ Aspartic acid the effect Missense, under sequence ID: MH373640.1, as shown in Figure (4). Compatibility of 99% in Gene Bank of *exoU* gene as shown in figure (5) under sequence ID: CP050334.1 having one Transition G to A in code GGC\GAC amino acid Glycine\Aspartic acid and Missense substitution, as well one Transversion A to T the code AAG\ATG amino acid change Lysine to Methionine the effect Missense. Amplification of the *toxA* gene for *P. aeruginosa* with two Transvertion A\C, C\G and T\G, code GAC>GCC, CTG>GGG, and CTG>GGG amino acids respectively Aspartic acid > Alanine, and Leucine> Glycine predicted Missense impact, under sequence ID: CP054472.1Compatibility of 99% in Gene Bank of *toxA* gene (Figure 6). Compatibility of 99 % in Gene Bank of *toxA* gene as shown in Figure (7) under sequence ID: CP054472.1 having three Transversion T\A, C\A, and T\G, that code CAT\CAA, CTG\AAG, CTG\AAG amino acid Histidine\ Glutamine, and Leucine\ Arginine the Missense substitution.

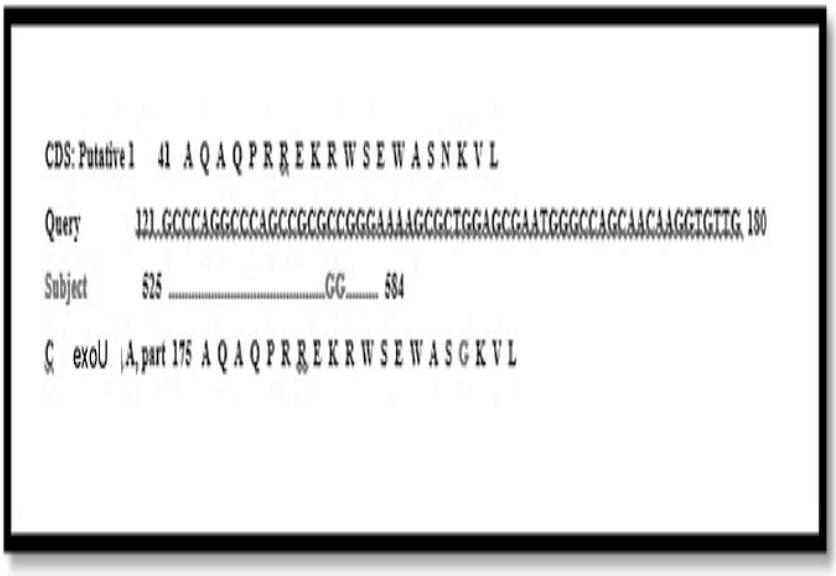

Figure (3): Alignment analysis of *exoU* gene of *P. aeruginosa* with Gene Bank of NCBI. Query represents of the sample; Subject represents a database of National Center Biotechnology Information (NCBI).

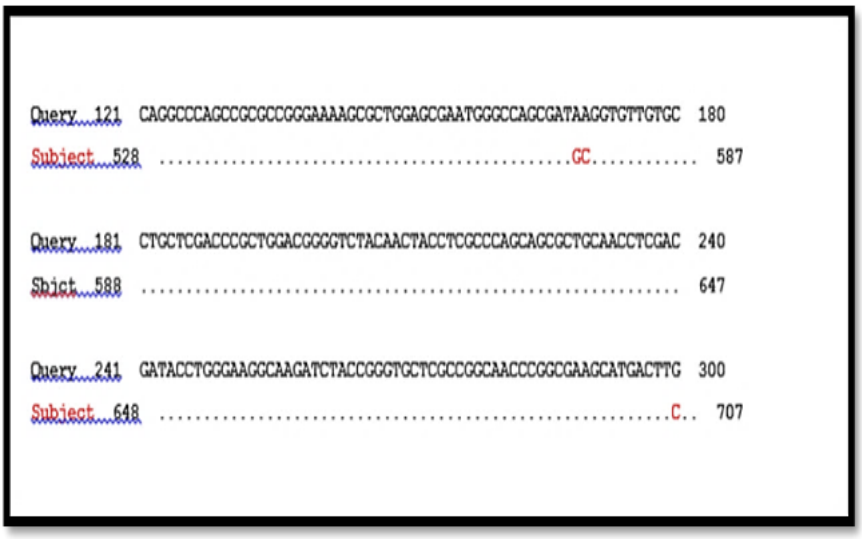

Figure (4): Alignment analysis of exoU gene of P. aeruginosa with Gene Bank of NCBI. Query represents of the sample; Subject represents a database of National Center Biotechnology Information (NCBI).

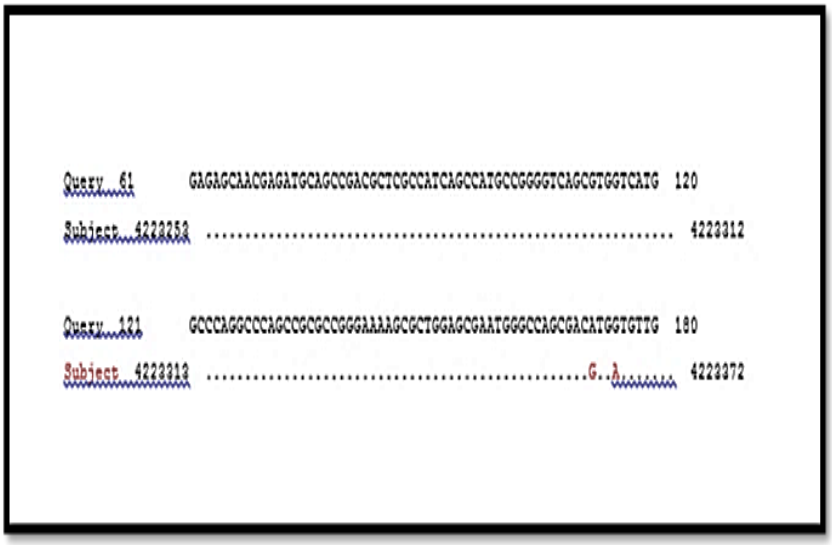

Figure (5): Alignment analysis of *exoU* gene of *P. aeruginosa* with Gene Bank of NCBI. Query represents of the sample; Subject represents a database of National Center Biotechnology Information (NCBI).

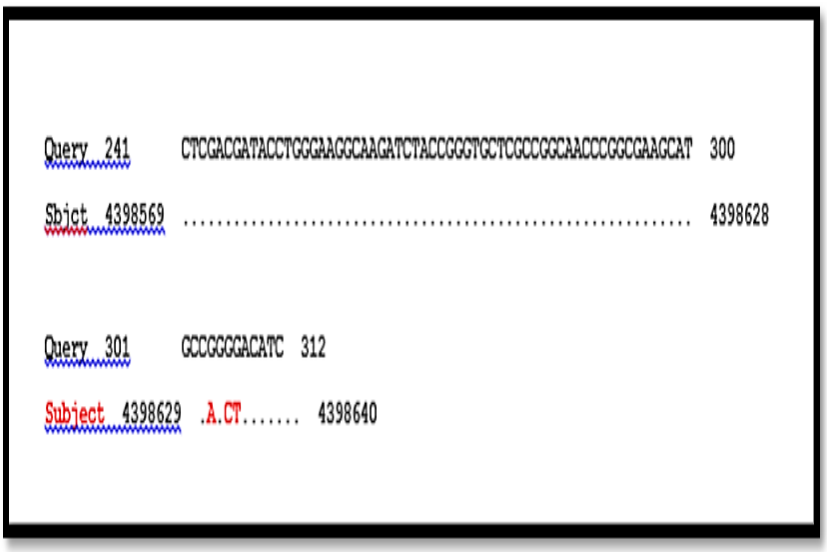

Figure 6: Alignment analysis of *toxA* gene of *P. aeruginosa* with Gene Bank of NCBI. Query represents of the sample; Subject represents a database of National Center Biotechnology Information (NCBI).

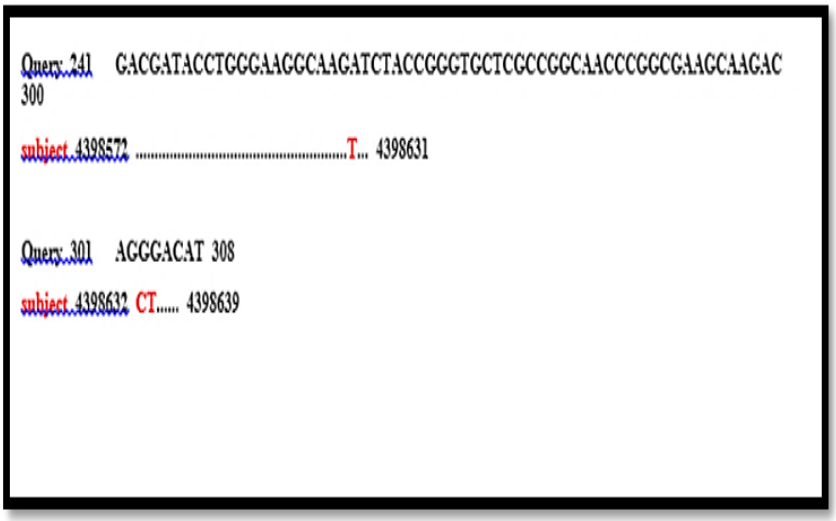

Figure (7): Alignment analysis of *toxA* gene of *P. aeruginosa* with Gene Bank of NCBI. Query represents of the sample; Subject represents a database of National Center Biotechnology Information (NCBI).

In their research results in Iraq, the researcher Neamah, 2017 indicated that the prevalence of the gene *tox*A in *P. aeruginosa* isolates from wound swabs was 100% and 92.8% in burn swabs **[**16**]**. In the other local and international studies, the percentages of *tox*A gene in *P. aeruginosa* isolates were 100% from swabs of burned Iraqi patients **[**17**]**. Yuosif *et al*., 2015 **[**18**]** found revealed that by using PCR of the bacterial DNA and the primer *tox*A with a size of 297 base pairs, and after conducting the electrophoresis, the product of this reaction was on the agarose gel at a concentration of 1.5% and examined with ultraviolet light, these bands appeared; the gene was found in all of the fifty isolates (100%) of Iraqi patients who were burned in Ramadi General Hospital/ Iraq **[**18**]**.

Conclusion

The *P. aeruginosa* which isolated from wounds and burns possessed virulence genes, *toxA* and *exoU* genes. The *exoU* and *toxA* genes having Transition, amino acid alteration and amino acid transformation.

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