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Prevalence of exoU/exoS genotype among*Pseudomonas aeruginosa* isolated from burn wound infections

[1]Maryam Ali Raouf , [2]Husam Sabah Auhim

[1,2] Department of Biology, College of Sciences, University of Baghdad, Baghdad, Iraq

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Abstract : *Pseudomonas aeruginosa* has been identified as the main causative agent responsible for severe infections in burn patients worldwide. This study aimed to investigate the prevalence of the exoU/exoS genotype in *P. aeruginosa* isolates collected from burn wound infections in Iraq. From January to April 2023, a total of eighty isolates of *P. aeruginosa*were obtained from patients with burn wound infections in two Iraqi hospitals (Teaching Baghdad Hospital and AL-Yarmok Hospital).The isolates were first identified using biochemical tests and then verified using molecular techniques, specifically by targeting the *16S rRNA* gene with specific primers. The exoU/exoS genotype was detected using conventional polymerase chain reaction (PCR) by specifically targeting two genes, *exoS* and *exoU*.The study found that burn wound infections contained four distinct genotypesof *P. aeruginosa*, and these genotypes exhibited substantial differences (P<0.001). The exoU-/exoS+ genotype exhibited a significantly higher prevalence of 75% (60 out of 80 isolates), whereas the exoU-/exoS- genotype had a comparatively lower prevalence of 5% (4 out of 80 isolates). At the same time, a lower percentage of isolates, 6.25 % (5 out of 80 isolates), was found to contain the *exoU* gene only (exoU+/exoS- genotype). Notably, 11 isolates (13.75 %) exhibited the presence of both *exoS* and *exoU* (exoU+/exoS+ genotype; a highly virulence isolate).bioinformatics analysis indicates that other bacterial species, such as *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Acinetobacter baumannii*, can produce ExoU toxin. The findingsprovide valuable insights into the genetic diversity of *P. aeruginosa* in burn wound infections, with the presence of highly virulent strains harbouring two genes (*exoS*and *exoU* gene).

Keywords:*P. aeruginosa*,exoU/exoSgenotype, Burn wound infections, *exoU* gene, *exoS* gene.

Corresponding author: (Email : husam.auhim@sc.uobaghdad.edu.iq)

Introduction:

Pseudomonas aeruginosa is a Gram-negative bacterium that is widely found in different environments and is known to be a major cause of healthcare-associated illnesses (1). *P. aeruginosa* is a major cause of disease and death in people with compromised immune systems and those who have experienced burns (2).The colonization

of this bacterium in healthcare facilities can result in a variety of severe illnesses in hospitalized persons, including septicemia, otitis, endocarditis, pneumonia, keratitis, as well as infections affecting the skin and soft tissues (3). Moreover, as a result of inherent or acquired resistance to numerous wide-ranging antibiotics, *P. aeruginosa* has emerged as a common infection in hospital environments. (4). Individuals with compromised immune systems, such as those who have undergone recent burn treatments, are especially susceptible to infection with *P. aeruginosa* (5). The death rate is higher among burn patients who are infected, particularly when the infection is caused by multi-drug resistant bacteria (MDR), as compared to burn patients who are not infected. The study and analysis of bacterial isolates related to burn wound infections have garnered considerable interest due to the frequent prevalence of multidrug-resistant *P.aeruginosa* infections in burn units (6).

Different protein secretion systems have the ability to release a protein either into the surrounding environment or into the host cell. The type III secretion system, also known as TTSS or T3SS, is the third type of secretion system found in bacterial cells. It appears like a needle in the structure of pathogenic bacteria(7), and it functions as a sensory transmitter to find eukaryotic organisms. It releases proteins that play a role in the spread of infection by bacteria. Exoenzymes or effector proteins are secreted from the bacterial cell into the eukaryotic host cell directly by this system, where they have various effects. This facilitates the survival, adaptability, and escape of the virus against the immune response. *P. aeruginosa* releases four different exoenzymes, namely S, Y, U, and T exoenzymes, through the T3SS (8). Bacteria that contain this mechanism produce exo Y and exo T in 100% abundance, while exo S and exo U are present in varying percentages. Nevertheless, recent isolates exhibited the presence of both *exoS* and *exoU*genes, with the other two genes, *exoY* and *exoT*. These isolates are categorized as extremely pathogenic isolates that possess all four toxins. (9).

The research indicates a correlation between the production of biofilms and the presence of the exoU/exoS genotype, as reported by Olaniran *et al*., (10). According to Derakhshan et al., (11), all biofilm-producing isolates included the *exoS* gene. The *exoU*gene is linked to higher resistance against certain antibiotics, and the *exoU* gene is more commonly present in multi-drug resistant *P. aeruginosa* strains (12). These findings indicate that the genotype is associated with additional virulence variables.

There is less knowledge regarding the distribution of exoU/exoS genotypes in burn wound infections in Iraq. Acquiring knowledge about the frequency of these genetic types will enable us to comprehend the importance of these genes in disease development and their prevalence in infection sites,leading to better strategies for treatment or prevention. Therefore, it is important to know the prevalence of exo U/ exoS genotypes in *P. aeruginosa* strains isolated from burn wound infection in Iraq.

Material and Methods

Specimens collection, bacterial isolation, and identification.

A total of 120 burn swabs have been collected from two hospitals in Baghdad, namely the Teaching Baghdad Hospital and AL-Yarmok Hospital, from January to April 2023. The swabs were culturedon MacConkey

agar and subsequently incubated for 24 hours at 37°C. For primary identification, microscopic examination was conducted together with the use of a selective cetrimide medium and biochemical tests such as oxidase and catalase. Additionally, incubation at different temperatures (4 and 42 °C) was performed. The identification was ultimately confirmed by molecular techniques that targeted a segment of the *16S rRNA* gene using specialized primers. The identifiedbacterial isolates were cultivated and stored at 4°C for subsequent examination.

Oligonucleotide primers

The process of designing oligonucleotide primers was conducted using Serial Cloner 2-6-1 and Amplifx software through computational methods. Initially, the sequences of the target genes were downloaded from the National Center for Biotechnology Information (NCBI) website. The accession number for the *16S rRNA* gene was PP448158.1, whereas the accession numbers for *exoS* and *exoU* were X99471.1 and KX641459.1, respectively. The primers have been manufactured by Macrogen Company and supplied in a lyophilized form.For the *16S rRNA* gene, the primer pair was16S Pseud-F:AGGCCTAACACATGCAAGTCGA ;16S Pseud-R:GGTTAGACTAGCTACTTCTGGA GC, with an amplicon size (1400bp), for the *exoU gene*, the primer pair wasExoU-F:CCGTTGTGGTGCCGTTGAAG, ExoU-R:TCATGTGAACTCCTTATTCCGC C,with an amplicon size (800bp),for the *exoS* gene, the primer pair wasExoS-F: GCGGACCTGAATCGCGCTCT,ExoS -R:CGTACATCCTGTTCCTGGACC, with an amplicon size (500bp).A stock solution of primer (100 pmol/µl) was prepared by dissolving the lyophilized primer in an appropriate amount of nuclease-free water,following the instructions provided by the manufacturer. Then, the working solution (10 pmol/µl) was prepared by diluting the stock solution tentimesand kept at -20 C in the freezer. The Tm Calculator website developed by Thermo Fisher Scientific (13) was utilized to determine the annealing temperatures for the primers.

Bacterial genomic DNA extraction

This study combines and adapts the boiling method described by Omar *et al*. 2014(14) and the colony PCR technique used by Auhim,2019 (15) for whole-bacterial genome extraction. In the present experiment, a single bacterial colony was transferred into a 5 ml nutritional broth and incubated for 24 hours at 37°C. Next, cells were collected by centrifugation at 13000 rpm for 15 minutes. The recovered cells were washed twice with 1 ml of distilled water and resuspended in another 1 ml. The cells were then standardised to achieve an optical density (O.D) value of 1 at a wavelength of 600 nm. Subsequently, an amount of 0.3 ml was taken from the standardised bacterial culture and transferred into an Eppendorf tube. The cells were subsequently collected by centrifuging for 15 minutes at 13000 rpm. Following this, the cells were resuspended in 0.75 ml of Tris-EDTA buffer (TE), which were subsequently subjected to boiling at 100°C for 10 minutes. The denatured proteins and cellular debris were removed from the genomic DNA using centrifugation for 20 minutes at 13000 rpm. The genomic DNA-containing supernatant was employed as a direct DNA template in PCR without additional purification, with a supernatant-to-PCR reaction mixture ratio 1:9.

Amplification of genes by polymerase chain reaction (PCR)technique

The conventional PCR technique was used to amplify particular regions of three target genes: the *16SrRNA* gene for molecular identification of bacteriaand the *exoS* and *exoU* genes to determine the frequency of the exoS/exoU genotype in *P. aeruginosa* isolates. Go Taq G2 Green Master Mix was utilised for gene amplification, and the PCR reaction mixture was prepared in a total volume of 100µl; the mixture contains 50µl of Go Taq G2 Green Master Mix (2X),5µl of each forward and reverse primer (10 µM/ µl), and 40µl ofnuclease-free water, then aliquoted 9 µl into 10 sterile PCR tubes, and 1 µl of DNA-containing supernatant from each bacterial isolate was added as a DNA template. The PCR reactions were conducted using an Applied Biosystems thermocycler with the conditions illustrated in Table 1.

Table (1): PCR conditions for gene amplification

PCR conditions					
Initial denaturation	30 reaction cycles			Final Extension	Hold
	Denaturation	Annealing	Extension		
5min 95˚C	30 s 95˚C	30 s X˚C*	50 s 72˚C	5 min 72˚C)	5min 4˚C
* X= Annealing temperatures used according to the primer pairs, 55˚C for *16S rRNA* and*exo S* gene, 57˚C for*exo U* gene					

Agarose gel electrophoresis

The PCR products were analysed using agarose gel electrophoresis, using 2% (w/v) agarose gel. The agarose gels were prepared by dissolving 2 g of agarose powder (Promega, USA) in 100 ml of 1X TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.8) using a microwave. After cooling to approximately 50-40 °C, the gel was then enhanced with 4 µl of ethidium bromide (10 mg/ml, Promega, USA). Subsequently, the gel was poured into a gel tray and left to solidify at room temperature (20-25°C). A volume of 5 µl of each sample was subjected to analysis with a volume of 3 µl of a DNA ladder (100 bp DNA, Promega, USA). The electrophoresis procedure was conducted at a voltage of 75 volts for 60 minutes. The DNA bands were detected using ultraviolet (UV) light in a gel imaging device (Fisher Scientific, UK).

Bioinformatic analysis of *Exo S* and *Exo U* genes

The sequences of each gene (*exoS* and *exoU*) were submitted separately in the BLASTN tool in NCBI for similarity searching and analysis of the distribution of these genes among *P. aeruginosa* and other possible bacteria.

Statistical analysis

All features appeared as frequencies and percentages, and the Pearson-Chi-square test was detected to reveal significant differences in percentages. $P \leq 0.05$ was measured as significant. The data were analyzed using SPSS v. 22.0 and Excel 2013 statistical software.

Results and discussion

Primary bacterial isolation and identification

A total of 80 *P. aeruginosa* isolates (66.6%) were isolated from 120 patients who were diagnosed with burn wound infections in Iraqi hospitals compared to

(33.32%, 40\120) of other microbial growth with significant differences ($p<0.05$). The primary identification of *P. aeruginosa* isolates was based on culturing characteristics on selective and differential medium, microscopic examination, and a few biochemical tests, as illustrated in Table 2.

Table (2): Cultural characteristics and biochemical tests of *P.aeruginosa* isolates.

Tests	Result
Growth on MacConkey Agar	Lactose non-fermenter, pale colony
Growth on Cetrimide agar	A present fluorescent green colour with growth
Gram stain	Gram negative, Bacilli
Oxidase test	Positive
Catalase test	Positive
Grow at 42°C	Positive
Grow at 4°C	Negative

Primary identification is considered an essential step to isolate the bacteria from the infection sites, and this identification helps to neglect other uninteresting bacteria that may be involved in infections, leading to saving time and material, especially as many different bacterial species can cause the infection in burn wounds (16,17). However,molecular identification based on the amplification of a specific gene is more reliable than other identification methods and is widely employed for pathogen detection and identification (18),for this reason, all isolates were subjected to molecular identification by targeting a segment of the *16S rRNA* gene (1400 pb) that shares 100% identity with *P. aeruginosa* using specific design primers. Four sets of results are illustrated in Figure 1, each representing a different group of isolates (1 to 20, 21 to 40, 41 to 60, and 61 to 80). The consistency of the banding patterns across all samples, as shown in Figure1, indicates the existence and successful amplification of the target *16S rRNA* gene, confirming the identity of the isolates as *P. aeruginosa*. This demonstrates the reliability and precision of this molecular technique in bacterial identification.This technique provided a high level of accuracy in confirming the identity of the bacterial isolates (19).

The findings of this study showed that the isolation percentage of *P. aeruginosa* (66.66%) from burn wound infections was higher when compared to international comparable investigations; in Ethiopia (12.86%)(20), Tanzania (12.6%) (21), Kenya (13.7%) (22), China (21%) (23), Pakistan (24.9%) (24), as well as Yemen (46.5%) (25). On the other hand, local investigations showed different ranges of isolation percentages. Polse et al., 2023(26), Anwer 2023(27), and Mohammed and Wasan 2022(28) have reported approximately similar isolation percentages of *P. aeruginosa*, which were (64.54%) in Duhok/Iraq, (67.1%), and (60.7) in Baghdad, respectively. At the same time, other studies reported different lower percentages: 11.4% (29), 17.78% (16), 30.05% (30), and 40% (31).

According to Abdi *et al*. (20), there could be several factors contributing to these variations in *P. aeruginosa* separation instances from burned areas between investigations: (i) the kind of selection criteria, (ii) the application of selective/differential mediums for cultivating the selected organism, (iii) collection protocols, and (iv)

differences in places of health care presenting establishments, which involves the existence or lack of aimed focuses for processing burn injuries. (iiv) personal hygiene and regional climatic conditions.

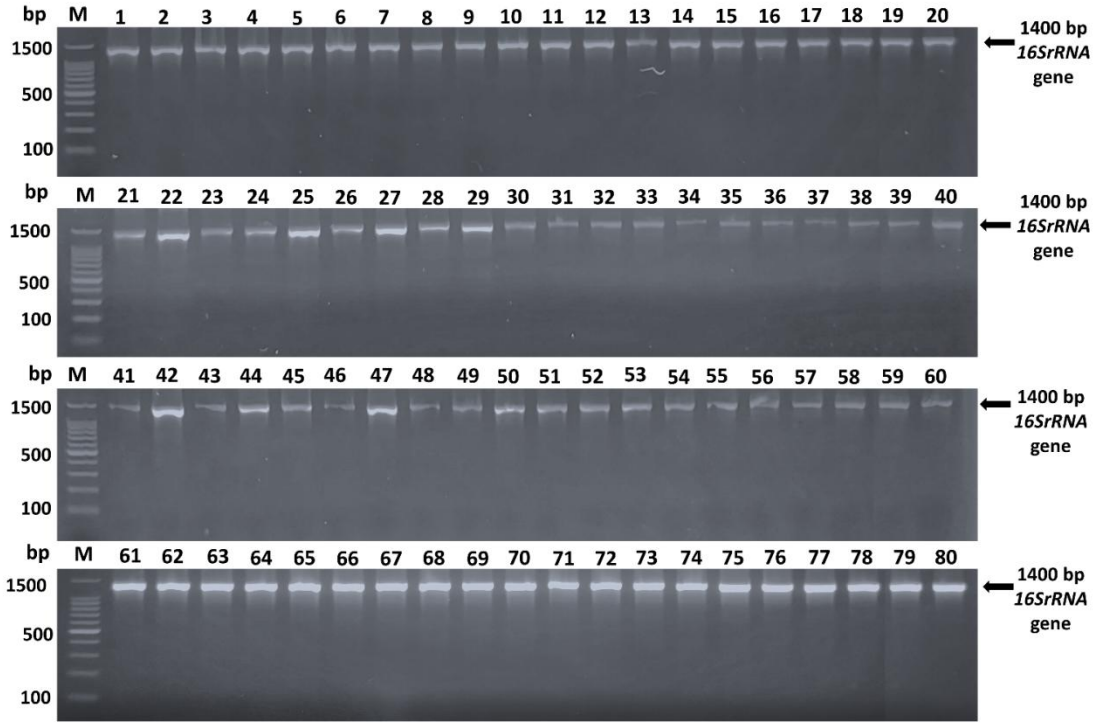

Figure (1): Agarose gel electrophoresis (2%) of PCR amplicon showing a segment of *16S rRNA* gene (1400 bp) amplification for *P. aeruginosa* identification. Numbers 1 to 80 correspond to *P. aeruginosa* isolates. M corresponds to the DNA marker (100 bp).

Molecular detection of exoU/exoSgenotype amongst *P. aeruginosa*

Tow genes were targeted to determine theexoU/exoSgenotype, which were *exoU* and *exoS*genes. The results showed a variable distribution of the *exoU* and *exoS* genes among the bacterial isolates under study with significant differences (P<0.001), as shown in Figure2. Based on the results of gene detection, the 80 bacterial isolates were categorised into four genotypes, as shown in Table 3. The first genotype (exoU-/exoS+), which was the highest one, was found in 75% of the bacterial isolates. In contrast, the second genotype (exoU-/exoS-) wasrelatively rare, found in only 5% of the bacterial isolates, indicating a lack of both key virulence factors (*exoU* and *exoS* genes). The third and most important one was the (exoU+/exoS+) genotype, indicating the presence of both virulence factors, which was observed in 13.75% of the isolates. Lastly, the fourth genotype (exoU+/exoS-) was detected in 6.25% of isolates.

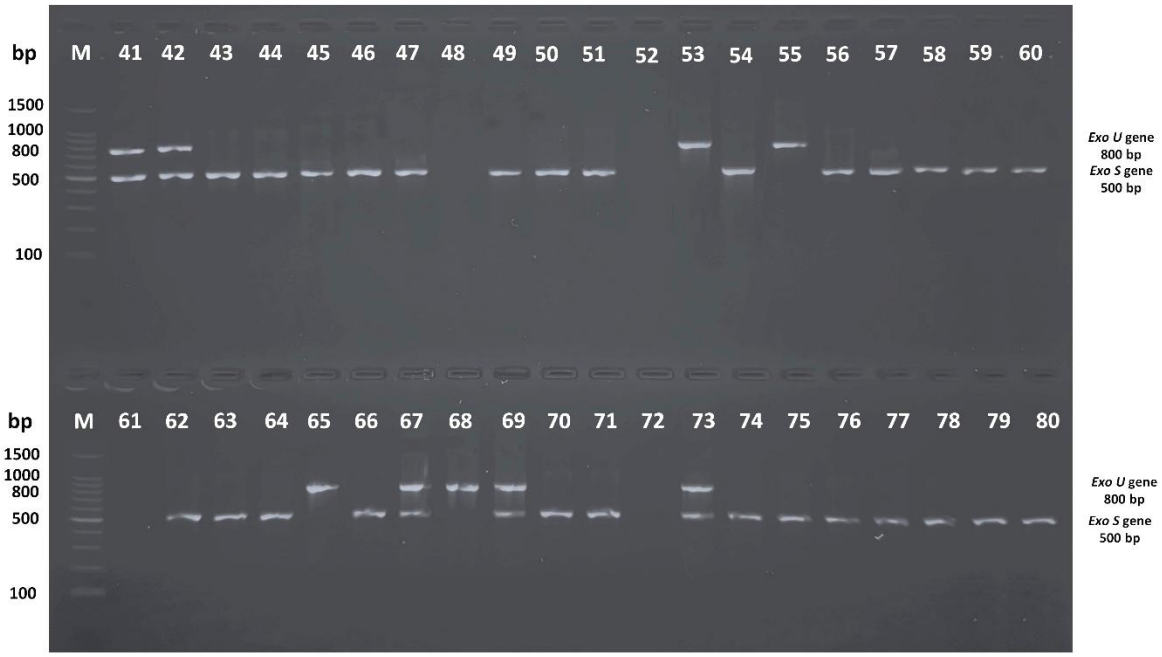

Figure (2): Agarose gel electrophoresis (2%) of PCR amplicon showing amplification of a segment of *exoU*gene (800 bp) and *exoS* gene (500bp) for *P. aeruginosa*. Numbers 1 to 80 correspond to *P. aeruginosa* isolates. M corresponds to the DNA marker (100 bp). Each lane in the gel represented a combination of separated PCR products for both *exoU* and *exoS* PCR products.

Table (3): Distribution and percentage of (exoU/exoS) genotype in *P. aeruginosa* isolates

Genotype	Number of isolates	Percentage
ExoU-/ExoS+	60	75%
ExoU+/ExoS+	11	13.75%
ExoU+/ExoS-	5	6.25%
ExoU-/ExoS-	4	5%
Total isolates	80	P<0.001***

Locally, most investigations focused on the detection of a single gene (*exoS* or *exoU*gene) rather than two genes to determine the genotype (32,33). Therefore, for comparison, the existence of each gene was recalculated regardless of genotype classification, as illustrated in Table 4.

Table (4): Distribution and percentage of *exoU* and *exoS* genes in *P. aeruginosa* isolates

Gene	Number of isolates	Percentage
ExoS+	71	88.75%
ExoU-	64	80%
ExoU+	16	20%
ExoS-	9	11.25%
Total isolates	80	P<0.001***

The current study's findings were in line with most of the local and international investigations, which showed that the *ExoS* gene recorded a high percentage of distribution among *P. aeruginosa* isolates. Locally, Asmaa et al.,(34) reported that 100% of *P. aeruginosa* isolated from burns harboured the *ExoS* gene. Another study conducted by Samah et al. (35) found that 40% of *P. aeruginosa* obtained from burns harboured the *ExoU* gene. According to Noor et al. (32), the *ExoU* gene was present in 60% of *P. aeruginosa* isolates obtained from burns. Samir and Mahde (36) reported that 56.25% of *P.*

aeruginosa isolates obtained from burns and wounds harboured the *ExoU* gene.

Another study by Anmar et al., (33), who isolated the *P. aeruginosa* from different clinical sources, found that *exoS* and *exoU* genes were present in 90.47% and 60.31% of *P. aeruginosa* isolates, respectively. Internationally, the *exoS* gene showed a higher prevalence than *exoU* in *P. aeruginosa* isolated from different clinical sources. A study conducted by Abozahra et al., (37) found that *exoS* and *exoU* genes were present in 57% and 43% of *P. aeruginosa*isolates, respectively. An investigation conducted by Noha revealed that the *exoS* gene was more prevalent (63%) among the isolates compared to the *exoU* gene (18%)(38). According to Wang et al., (1) found that *exoS* and *exoU*genes were present in 78.46 % and 16.26% of *P. aeruginosa* isolates, respectively.

Regarding the genotype, the exoU-/exoS+ genotype scored higher than other genotypes. The finding of the study conducted by Horna et al., (39) revealed that 77.24% of *P. aeruginosa* isolated from different clinical sources were exoU-/exoS+ genotype, while 22.75% were exoU+/exoS+ genotype. These results agreed with the current study's findings that showed that 75% and 13.75 of *P. aeruginosa* were exoU-/exoS+ genotype and exoU+/exoS+ genotype, respectively.

The high prevalence of the *exoS*gene and exoU-/exoS+ genotype in *P. aeruginosa* isolatescould be explained by the importance of ExoS toxin as an invasive virulence factor, which is a bifunctional toxin with GTPase activating protein and ADP-ribosyltransferase activities. These activities lead to disruption of the actin cytoskeleton and apoptosis-like cell death (9). However, isolates of *P. aeruginosa* were categorized as having either invaded or cytotoxic phenotypes based on toxins produced by the type III secretion system. Bacteria with the *exoS* gene are invasive, whereas strains with the *exoU* gene are cytotoxic (40,41).

The *exoU* gene encodes the ExoU toxin, which is a strong cytotoxin with phospholipase A2 activity that results in rapid lysis of many mammalian cell types. It has already been documented that ExoU causes damage to monocytes and epithelial cells (42). According to reports, eliminating the *exoU* gene significantly decreased *P. aeruginosa's* pathogenicity and major pathologic problems in the respiratory tract (43). ExoS is more common than ExoU. Despite being less common, ExoU-producing strains have been reported linked to high death rates (44).

The exoU+/exoS+ genotype, which is present in 13.75% of bacterial isolates under investigation, is considered a highly virulence one, as the ExoU and ExoS are thought to be the most important virulence factors of *P. aeruginosa*. This genotype is known as invasive and cytotoxic, as described above. Even though this genotype represents a lower percentage compared with other studies that showed a high percentage ; 38.46% (45) and 61.2% in isolates obtained from cystic fibrosis patients (46), this is a concerning sign that a very detrimental strain is spreading in burn wound infections in Iraq.

Regarding the exoU-/exoS- genotype,which represented 5% of bacterial isolates. These isolates lack the type III secretion system, as indicated before, and bacteria that have a type III secretion system must secrete ExoU, ExoS, or both (47). However, bacteria lacking a type III secretion system employ another toxin that facilitates their pathogenicity, which is Exolysin (ExlA) (48). The exoU-/exoS- genotype

could have an (ExlA) instead of type III secretion system toxins.

The capacity of isolated to adjust effectively to the environment that exists in the area of infection may account for the heterogeneity in the arrangement of virulence genes within them. Many variables, such as the degree of drug resistance, virulence gene alterations, and infection site, might influence the prevalence of a particular virulence factor (49). This explains why the existence of type III secretion system toxins varies between the environmental and pathogenic isolates as well as amongst pathogenic isolates isolated from different clinical sources (40,50).

Bioinformatic analysis of *exo S* and *exo U* genes.

Bioinformatic tools are extensively employed for the analysis of DNA and amino acid sequences. These tools are used to search for similar targets, align sequence, design primers, predict secondary structure, construct three-dimensional structures (models and actual structure), validate structure, and predict protein functions, and protein-protein intreactions (51,15).The results of the search amongst 200 whole genomes of *P. aeruginosa* revealed that none of the isolates under analysis have four toxins. However, *P. aeruginosa*, which has the type three secretion system, has been reported to produce four toxins (ExoY ,ExoT, ExoS, and ExoU). Typically, no strain expresses more than three toxins. ExoS and ExoU appear to be nearly mutually exclusive, whereas ExoY and ExoT are encoded by almost all pathogenic strains (52,53,47). The new isolates appeared to have both genes (*exoS* and *exoU*) in addition to the other two genes, *exoY* and *exoT*. These isolates are classified as highly virulent isolates containing the four toxins (9).

Interestingly, even though these toxins were produced only by *P. aeruginosa*, as established by many researchers, the search results revealed that other bacterial species contain ExoU toxin with a sequence identity of 100%. These bacteria were *Klebsiella pneumoniae* (accession number: MBM0045430.1); *Enterobacter cloacae* (accession number: SAJ32780.1); *Acinetobacter baumannii* (accession number: SST10122.1). These results indicate the ability of other bacterial species to acquire ExoU toxin using one of the methods for transferring genetic material, especially the presence of *exoU* within a mobilizable genomic island 54). This is a worrisome indication of the dissemination of highly virulent factors amongst pathogenic bacteria.

Conclusion

Different genotypes of *P. aeruginosa* are present in burn wound infections in Iraq based on the presence of Type III secretion system toxins,with the presence of highly virulent strains harbouring two genes (*exoS* and *exoU* genes).The exoS+\exoU- genotype (invasion genotype) is more prevalent in burn wound infections in Iraq than in other genotypes. This could be because burn wounds are soft, damaged tissue, which makes the invasion bacteria easier to spread.Other bacterial species, such as *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Acinetobacter baumannii*, can produce ExoU toxin, as bioinformatics analysis indicates. While our study provides valuable insights, further research is needed to fully understand the potential relationship between genotypes and other virulence factors in *P. aeruginosa*.

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Conflict of Interest

The authors declare that they have no conflict of interest

Authors' Contribution Statement

M.A.R and H.S.A. contributed to the research design and implementation, results analysis, and writing of the manuscript.

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