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Molecular Characterization and Differential Expression of Virulence Genes in Pseudomonas aeruginosa Isolates from Diverse Clinical Sources

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Annotation: Background: Different types of illnesses, like burns, wounds and urinary tract infections, caused by pathogen *Pseudomonas aeruginosa*. This is because having a wide range of virulence factors, which allow it to harm the host's immune defenses and cause damage.

Objectives: The aim of this study was to isolate *P. aeruginosa* from different clinical sources and investigate molecular detection of virulence genes and the variation in gene expression of *exoT*, *exoU*, in *P. aeruginosa* isolates.

Materials and Methods: Collected eighty samples from various clinical sources such as burns, wounds and urine infection from patients attending to AL-Diwaniyah Teaching Hospital and Burns Hospital in AL-Diwaniyah governorate, from period 1/10/2024 to 1/1/2025. The samples were cultivated on culture media and depending on microscopic, morphological traits, biochemical tests and Vitek system the isolates were identified. Technique polymerase chain reaction (PCR) was used to molecular detect of (*exoT*, *exoU*) genes and technique Real Time PCR examined the gene expression of (*exoT*, *exoU*) genes in *P. aeruginosa* isolates.

Results: The results revealed that *P.*

aeruginosa isolates constituted 12 (15%) of the total samples, distributed among five isolates from burns and five from wounds, representing 16% and 14%, respectively, and *two* (13%) isolates from urine samples. The results showed that all isolates contained *exoT* and *exoU* genes at a rate 100%. Significant differences were observed in the level of gene expression of the genes, as it was found in *P. aeruginosa* isolates from wounds, *exoU* gene showed the highest level of gene expression (22.53±14.21a) compared to the rest of the clinical sources. In contrast, *exoT* gene expression level was found to be relatively stable across all isolates.

Conclusion: *P. aeruginosa* is a pathogen that causes urinary tract infections, burns, and wounds. Virulence genes *exoT*, *exoU* were present in all isolates, but the most highly expressed gene in wound samples was *exoU* compared to the rest of the clinical sources.

Keywords: Pseudomonas aeruginosa, Clinical sources, Virulence genes, Type III secretion system, Gene expression, exoT, exoU.

Introduction

Gram-negative and opportunistic *P. aeruginosa* is a pathogenic bacterium that is widely found in many different environments, particularly hospitals, and is a serious threat to human health; it is a significant contributor to acquired - hospital infections. Especially in patients with severe illnesses and compromised immune systems, it causes a variety of infections, such as severe burns, wounds, urinary tract infections (UTI) [1]. In order to adapt the unfavorable environment of the host's and promote infection and disease, *P. aeruginosa* can secrete a numerous virulence factors [2]. Type III secretion system (T3SS), which takes shape molecular needle hollow and transports toxins from the bacteria into the cytosol of host cell's, it is one of *P. aeruginosa* most significant virulence factors. It works with excessive accuracy, changing host cells and manipulating [3]. To expression of virulence, *P. aeruginosa* uses this system that contains a collection of external enzymes, such as *exo T*, *exo A*, *exo U*, and *exo S* which are transferred directly to the target cell [4].

ExoU and *exoT* shown to contribute to the invasion of bacteria through toxicity to cells and for host immune responses [5]. Also *exo T* contributes to the spread of harmful bacteria as its enzymatic activities weaken immunity, prevent phagocytosis, and damage epithelial cells [6]. It was discovered that *ExoT* enzymatic activity delays wound healing, which increases *P. aeruginosa* opportunistic potential by greatly enhancing its capacity to exploit holes in the mucosal barriers [5]. Additionally, it was discovered that *P. aeruginosa* expression of the enzyme *exoU* makes it more pathogenic, which leads to severe infections [7], this is due to the enzyme's potent activity against phospholipases, which causes neutrophils, macrophages, and epithelial cells to rapidly lyse and necrotize, a reduction in neutrophils weakens the immune system and increases the host's vulnerability to infection by these harmful bacteria [8]. It is

regarded as a pathogenic cytotoxin that destroys epithelial cells and macrophages by causing necrosis. Since it is secreted and expressed at a rate of 20% in isolates from clinical sources, it is regarded as one of the genes pathogenic present in genome of *P. aeruginosa* and encoded in pathogenicity islands-2 [9].

The environment from which *P. aeruginosa* was isolated, it can often affect on the highly regulated expression of virulence factors in the bacteria. Due to the importance of *P. aeruginosa* and the pathogenicity it causes in a variety of diseases, this study aimed to isolate *P. aeruginosa* from different clinical sources and to molecularly investigate virulence genes associated with the type III secretory system, specifically the genes (*exoT*, *exoU*), in addition to the investigating gene expression of *exoT*, *exoU*, in *P. aeruginosa* isolated from different clinical samples. To understand the possible interactions between these components and better understand to the mechanisms that enable *P. aeruginosa* to adapt with different clinical cases.

Materials and Methods

Collecting of samples

Between 1/10/2024 to 1/1/2025, collected eighty samples from various clinical cases of patients who attending to Al-Diwaniyah Teaching Hospital and Burns Hospital in Al-Diwaniyah governorate. These samples were dispersed: Thirty samples of burns, thirty-five samples of wounds, and fifteen samples of urine from patients with UTIs. Samples of urine collected by using containers sterile, and samples from patients with wounds and burns were obtained using swabs cotton sterile. Following that, the samples were brought to the lab for bacterial culture.

Bacterial isolation and phenotypic identification

For primary isolation, samples were cultivated on culture media such as blood agar and MacConkey agar, which made in accordance with the manufacturer's instructions (Himedia/India). According to [10], all samples were cultivated using the planning method, and incubated in (37°C) for (24 hour). Then, *P. aeruginosa* isolates were diagnosed based on the phenotypic traits on culture media, such as colony shape, size, height, and lactose fermentation capacity. In addition to microscopic diagnosis, Gram staining was utilized to identify Gram-negative isolates by analyzing morphology of bacterial cells and how they interacted with the stain. The ability of *P. aeruginosa* to grow on Simmons citrate agar medium was another factor used to make the diagnosis, a number of biochemical tests were conducted, such as the catalase, oxidase, and indole tests according to [10, 11].

Primers

Iraqi Scientific Research Company Limited (ISRCL) developed primers for the detection of *exoT* and *exoU* genes and investigated expression of genes using (Real-time PCR) technique in *P. aeruginosa* isolates. While housekeeping genes were designed based on [12]. Using Primer3 plus software and NCBI-Genbank sequences, as shown in tables (1, 2).

Table (1): Primers gene of PCR detection

Primers	Sequence		Product Size
exoT	F	CTTCGTGGCGATCATCGAGT	504 bp
	R	TTTACCTCGCTCTCTACCGC	
exoU	F	GACAGATCGCTACGCATCCA	328 bp
	R	CCGCCATCCTGGAATTCTGT	

Table (2): Gene primers of qPCR detection

qPCR Primer	Sequence		Product Size	NCBI Reference
exoT	F	CAGCATGTACTCAGCGCAAG	71 bp	OQ302274.1

	R	TTGTCCATTACCGCATGCAG		
exoU	F	ACCCTTTCCGACAAGATGGATC	110 bp	KX641462.1
	R	AAGCCCTTTTTCAGCGATGC		
Housekeeping	F	TATGCACCCGCGTATACACC	110 bp	NC_002516.2:c4772127-4771756
	R	ACCACCGATGTACGAGGAAAC		

Extraction DNA

The Presto™ Mini gDNA Kit, was used to extracted DNA from isolates of *P. aeruginosa* in accordance with the manufacturer's instructions (Geneaid, Taiwan) in order to detect virulence genes (*exoT*, *exoU*).

Making the PCR master mix

As directed by the manufacturer (USA, Promega), the GoTaq®Green PCR master kit was used to create the PCR master mix for virulence genes. A PCR thermocycler (BioRad/USA) was then used to perform PCR for the *exoT* and *exoU* genes.

Gel electrophoresis

Agarose gel 1.5% used to analyze results of genetic PCR. The apparatus electrophoresis was run in for 100 watts, 80 amperes at one hour, after the gel was submerged in Tris/Borate/EDTA buffer and the cover was fastened. After that, the gel was exposed to UV light, and a camera (Nikon, Japan) was used to take a picture of it in order to read the results.

Extraction of RNA

Extraction kit Easy-BLUETM total RNA (Intron, Korea) was used to extract RNA of *P. aeruginosa* isolates, process of extraction was done in compliance to the instructions of the kit's.

DNase I treatment

Based on directed by the manufacturer (Promega, USA) DNase I kit was used based on instructions that came with it, to use DNase I enzyme in process of extracted RNA and remove any remaining DNA.

RNA conversion to cDNA

After DNASE-I treatment of RNA samples, M-MLV from reverse transcriptase kit, of Korean company Bioneer was used to convert them into cDNA by the instructions of the kit.

Making a master mix for real-time PCR (qPCR)

Using SYBER green dye for amplification in real-time PCR system, qPCR master mix was prepare with master mix kit GoTaq® qPCR (Promega, USA). Following the instructions of kit, 5 µL of cDNA template (10 ng), 1 µL of forward and reverse primers, 10 µL of qPCR master mix, and 3 µL nuclease-free water added to create the qPCR mixture for target genes and housekeeping gene, for a final volume of 20 µL. Following preparation, the qPCR mixture was put into white plate qPCR tubes, mixed for five minutes in a vortex centrifuge (P-ABC/USA), and then put into the CFX96 Real-Time PCR apparatus (BioRad/USA).

qPCR Thermocycler Conditions

A Thermocycler (BioRad/USA) was used to determine the conditions for thermal cycling. The qPCR Thermocycler kit instructions were followed in determining the conditions. The primer annealing calculation conditions were determined using Optimase ProtocolWriterTM software. The procedures were as follows: Initial Denaturation (10 min at 95°C), denaturation (20 sec at 95°C for 40 cycles), annealing, extension, and detection (scan) (60°C for 30 sec at 40 cycles), and melting (65- 95°C for 1 cycle).

Analysis of gene expression data

The qPCR results and gene expression data for the virulence genes *exoT*, *exoU*, and housekeeping gene were analyzed using the equation (The CT Method Using a Reference Gene), by the information provided in [13].

Analysis of statistics

According to [14], SPSS version 27 used, and the threshold for statistical significance was chosen a p-value of 0.05. Findings were displayed as mean ± standard error, frequencies, and percentages; a p-value of less than 0.05, as determined by chi-square analysis of variance (ANOVA), was deemed statistically significant.

Results

Identification and isolation

A study aimed to isolate *P. aeruginosa* from multiple clinical cases and investigate the virulence factors that enable it to adapt to different environment. The isolation rates of *P. aeruginosa* from various disease cases are displayed in Table (3) and Figure (1). Three clinical cases involving samples from burns, wounds, and urinary tract infections were used to isolate *P. aeruginosa*. Out of the 80 samples, 12 isolates, representing 15% of the total number of samples These samples were distributed into five isolates from burns and five isolates from wounds, representing 16% and 14% respectively, while only two isolates were found, representing 13% of patients' urine samples.

Table (3) *P. aeruginosa* isolates according to sample source

Sample source	Number of samples	Number of *P. aeruginosa*	Percentage (%)
Burn	30	5	16
Wound	35	5	14
Urine	15	2	13
Total	80	12	15
Calculated X^2	0.112		
Calculated P value	0.946*		

* No significant difference at P<0.05

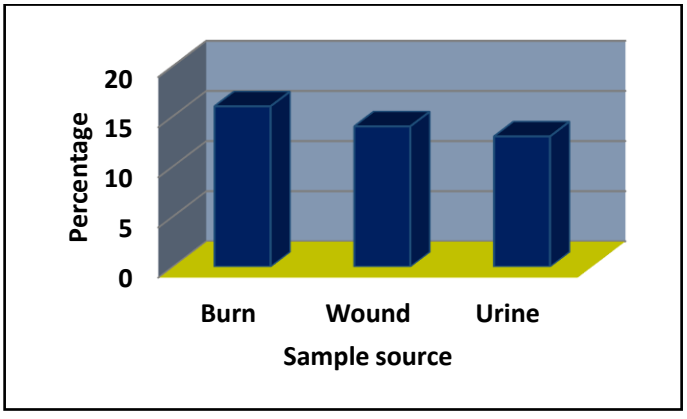

Figure (1): Disruption *P. aeruginosa* isolates according to clinical source

The diagnosis of every *P. aeruginosa* isolate on the solid culture media, the initial diagnosis was made using morphological and cultural traits. According to the diagnosis, *P. aeruginosa* colonies were small, smooth, and did not ferment lactose when they were on MacConkey medium. Hemolysis detection, by formation a halo transparent surrounding colonies on blood agar. All *P. aeruginosa* isolates were found Gram-negative rods upon microscopic analysis. All *P. aeruginosa* isolates tested positive for catalase, oxidase, and indole, according to the biochemical

test results. The growth test on Simmons citrate medium also yielded positive results, as evidenced by the medium's color changing from green to blue, which is regarded as a positive test result. Furthermore, the Vitek device's confirmatory diagnostic test result indicated that all isolates were, with a 99% probability *P. aeruginosa*.

Molecular Identification of the Virulence Genes *exoT* and *exoU*

All isolates of *P. aeruginosa* from different sources, such as burn, wound, and urine samples, contained the *exoT* and *exoU* genes at a 100% rate with a product size of 504 bp and 328 bp, respectively, according to the study's findings after genetically examining the presence of these genes using agarose gel electrophoresis (Figures 2 and 3). There was no difference among clinical sources of *P. aeruginosa* in containing these genes, which helps it cause disease and be more virulent.

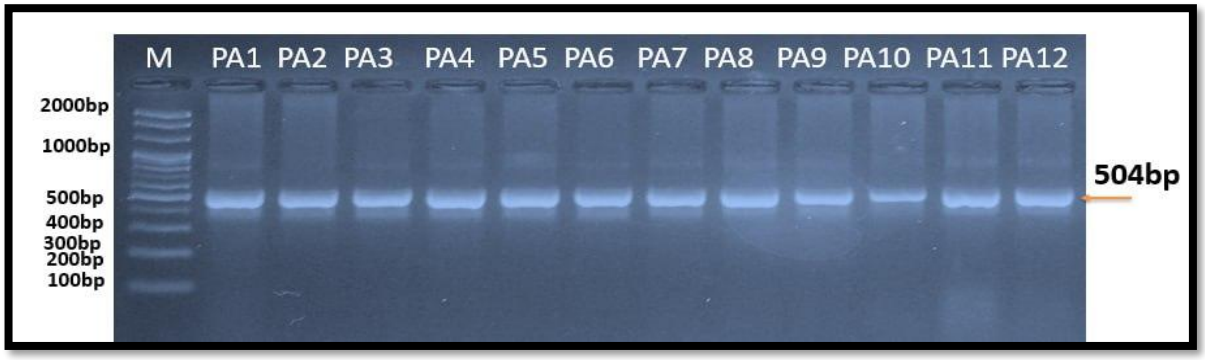

Figure (2): PCR detection of the *exoT* gene in *P. aeruginosa* isolated from various clinical sources with a product size of 504 bp (M: Marker ladder 100-2000 bp) is demonstrated by electrophoresis.

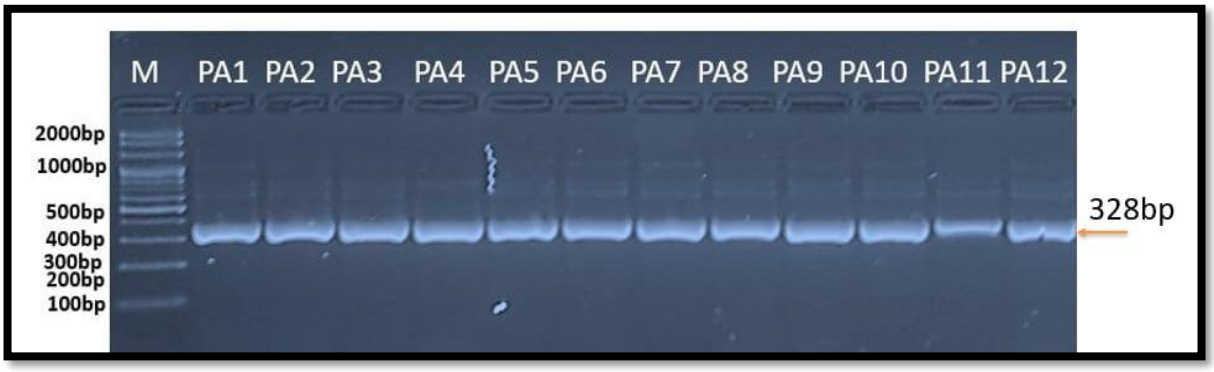

Figure (3): PCR detection of the *exoU* gene in *P. aeruginosa* isolated from various sources, with a product size of 328bp (M: Marker ladder 100-2000 bp) is demonstrated by electrophoresis.

Gene expression of virulence genes

Following the conclusion of the (RT-qPCR) run for virulence genes *exoT* and *exoU*, quantitative gene expression was assessed in comparison to the housekeeping gene. The results of the gene amplification were displayed as curves that displayed the actual amplification status. *P. aeruginosa* isolates from various clinical sources exhibited a significant variation in the level of gene expression for the virulence genes represented by *exoT* and *exoU* genes, as indicated by Table (4) and Figures (4 , 5) which display the outcome of the real-time polymerase chain reaction for the *exoT* gene. The *exoT* gene amplification results demonstrated a clear amplification curve in every *P. aeruginosa* isolate from various disease states (burn, wound, urine). Similarly, all isolates had a single and stable melting peak on *exoT* gene solubility curve. This indicates that the reaction was specific and that no non-specific dimers or other products

affected the reaction result. Demonstrate that *exoT* gene found in a comparatively close and constant gene expression level in all clinical cases. Urine samples had the highest *exoT* gene expression level, measuring (12.21±10.3a). While *P. aeruginosa* isolates from burn samples had lowest expression level (4.51±1.32a), isolates from wound samples had average gene expression levels of (9.91±3.34a). The statistical analysis's F value was 1.094 and the computed P value was 0.375, indicating that there were no appreciable variations in the *exoT* gene's expression between clinical cases.

As can be seen from Figures (6, 7) and Table (4), which display the outcomes of the (RT-qPCR) for *exoU* gene. The gene *exoU* was found in every isolate from various clinical sources, according to amplification results using (qPCR). Gene *exoU* amplification curve results, which appeared as a letter S, demonstrated successful and unambiguous DNA amplification, as can be seen from Figure (6). On the other hand, only one melting peak was visible in the melting curve analysis results, as can be seen in Figure (7). This proves that there were no other non-specific products and that the amplified product is specific. The results revealed notable variations in the levels of gene expression of *P. aeruginosa* isolates from various disease conditions. It showed that the highest expression level was in wound samples (22.53±14.21a), while urine samples showed average levels of gene expression (4.41±0.24a), *P. aeruginosa* isolates from burn samples had the lowest gene expression level, measuring 3.38±0.52a.

Table (4) *exoT* and *exoU* gene expression of *P. aeruginosa* isolates according to sample source (Mean ± SE)

Sample	*exoT* gene expression	*exoU* gene expression
Burn	4.51±1.32a	3.38±0.52a
Wound	9.91±3.34a	22.53±14.21a
Urine	12.21±10.3a	4.41±0.24a
Calculated F	1.094	1.154
Calculated P value	0.375	0.358

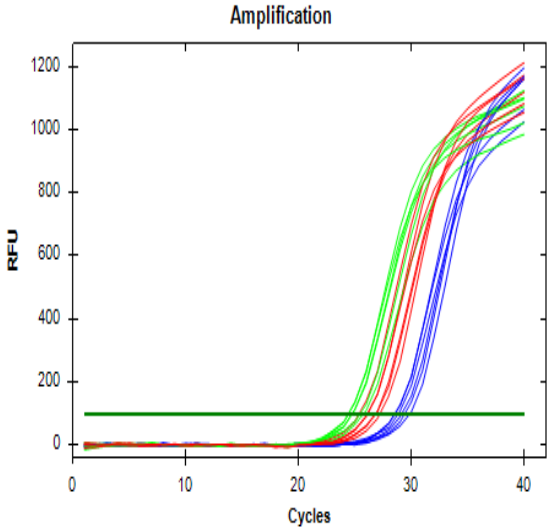

Fig4: qPCR plots of *exoT* gene in *P. aeruginosa* isolates. The green plots (urine), the red plots (wound) and blue plots (burn).

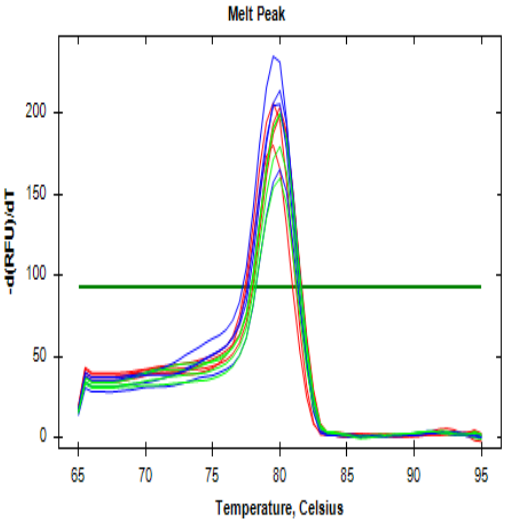

Fig5: qPCR melt peak of *exoT* gene in *P. aeruginosa* isolates. The green plots (urine), the red plots (wound) and blue plots (burn).

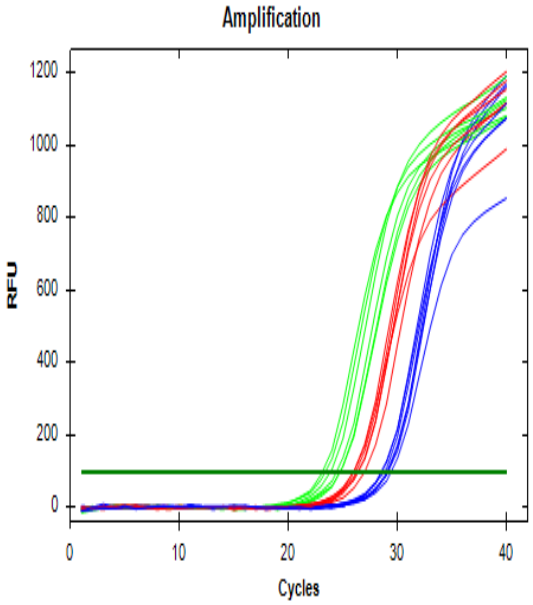

Fig6: qPCR plots of *exoU* gene in *P. aeruginosa* isolates. The green plots (urine), the red plots (wound) and blue plots (burn).

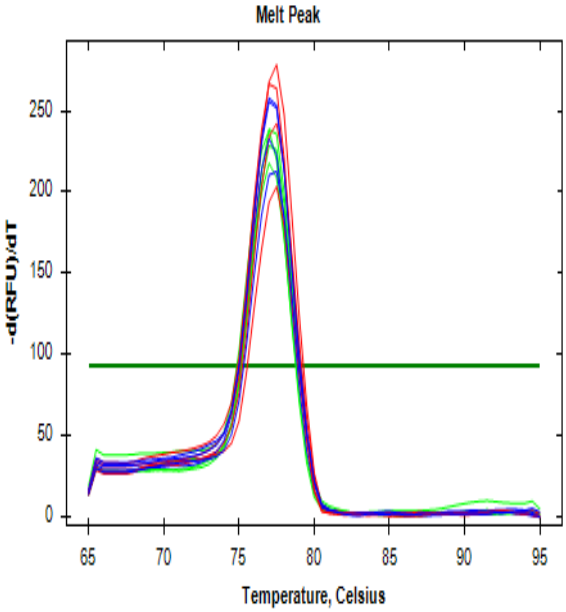

Fig7: qPCR melt peak of *exoU* gene in *P. aeruginosa* isolates. The green plots (urine), the red plots (wound) and blue plots (burn).

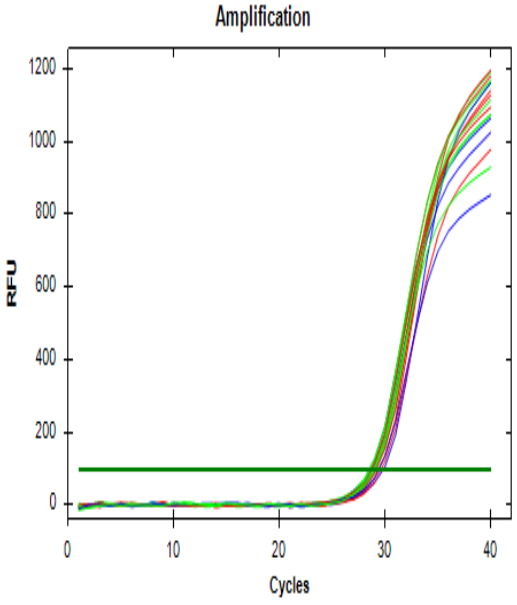

Fig 8: qPCR plots of houskeeping gene in *P.aruginsoa* isolates. The green plots (urine), the red plots (wound) and blue plots (burn).

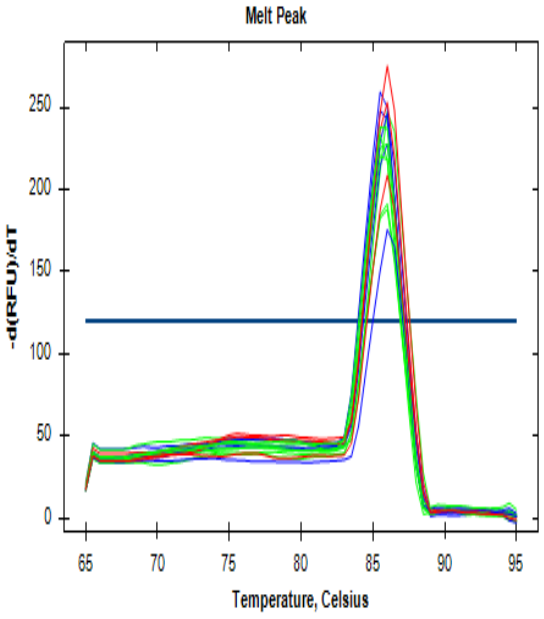

Fig 9: qPCR melt peak of houskeeping gene in *P.aruginsoa* isolates. The green plots (urine), the red plots (wound) and blue plots (burn).

Discussion

The objective of this research was to isolate *P. aeruginosa* from various disease states and examine the expression of its virulence factor genes. It is considered an opportunistic pathogen especially in patients have compromised immune systems, can cause severe burns and wound infections [15]. Following sample collection, bacterial isolation, and diagnosis using morphological and cultural traits, biochemical testing, and confirmatory diagnostic testing, it was

discovered that 15% of the samples were *P. aeruginosa*. These samples, which accounted for 16% and 14% of the total, were split between 5 burn samples and 5 wound samples, and only two isolates (13%) of urine samples were discovered from patients. These results were similar to those of [16], who found that the percentage of its isolation from burns was 19.8%. Conversely, our findings differed from those of [17], who discovered that the rate of isolation reached 64.55% from burn cases in Duhuk. Additionally, our results were not corresponding to [12], who reported that the percentage of this bacterium from wound infections was 26.4%, and the rate from UTI was 20.7%. The difference in isolation rates of *P. aeruginosa* compared to earlier studies may be due to the environment from which it was found and various method to collect pathological samples may have an impact on the density and appearance of bacteria *P. aeruginosa*.

According to molecular detection of *exoT* and *exoU* in *P. aeruginosa* isolates from burn, wound, and urine samples had 100% of these genes, presence of type III secretory system genes *exoT* and *exoU* in these isolates indicates they are clinically hazardous, were present in all isolates regardless of infection site and can participate in pathogenicity of this bacteria. This is corresponding to result of [18] who found relationship between the influences of *exo* genes and severity of infection. Our results were consistent to [19] who found a high percentage of *exoT* gene (96.13%) of *P. aeruginosa* isolates from disease cases, while our results differed with them, in the presence of *exoU* gene which reached 24.70%. Our results also agreed with [20], who found that *exoT* was present in 100% of isolates, and our results differed from [21] who found that *P. aeruginosa* isolates from burn and wound infections had 40% of *exoU* gene. Additionally, the results differed from [22], who found that *P. aeruginosa* isolates had 69.21%.of *exoT* gene.

Upon investigating the expression of virulence genes (*exoT*, *exoU*) possessed by this bacterium, it revealed that *P. aeruginosa* distinguishes by the ability to express these genes in various clinical cases, suggests that the bacteria can be compatible and adapt with different host environments, which help in infection. It corresponds to [23] who indicated that the genes causes virulence are associated with clinical sources of *P. aeruginosa* infection. As a result, investigating these genes is important to limit the spread of infection and the transfer of these genes between isolates. The results we obtained indicated showed that all isolates had consistent and regulated of *exoT* gene expression, despite the source of the infection. This may indicate that this gene is a major factor in *P. aeruginosa* pathogenicity. This corresponds to the findings of [24], which found that *exoT* contributes in causing pathogenesis and virulence of *P. aeruginosa*. According to [25] it is considered as an indicator of an epidemic of this bacterium that causes infection in a variety of clinical conditions. Additionally, results demonstrated that the *exoU* gene was expressed in every isolate, indicating that it is a significant virulence factor that is present in *P. aeruginosa* isolates from a variety of hospital-acquired clinical cases

The results also showed that *exoU* gene expression was elevated in isolates from wound infections, which can contribute to tissue damage, these findings are consistent with those of [21] who reported increased *exoU* gene expression in *P. aeruginosa* isolates from wounds. Due to the high phospholipase activity of this gene, which causes rapid tissue damage, high *exoU* gene expression in *P. aeruginosa* is associated with delayed treatment and increased severity of the disease [26]. The findings of the study also showed that the *exoU* gene was expressed in *P. aeruginosa* isolates of urine and burn samples. Although *exoU* gene is present in every isolate, Variations in the quantity of *exoU* may be the cause of variation in expression levels, expression levels of isolates from various clinical conditions differ; the higher expression, the more pathogenic and infectious. The *exoU* gene expression in *P. aeruginosa* is a major factor, which has increased pathogenesis in clinical cases [27], because they are considered virulence factors that contribute to incidence of infection in the host and increase in mortality, as a result of infection with in *P. aeruginosa* according to [28].

Conclusion

P. aeruginosa is a pathogenic causative agent of burns, wounds, and UTI, according to the study's findings. All *P. aeruginosa* isolates possessed the *exoT* and *exoU* genes, which confer virulence and pathogenicity, regardless of the source of the isolates. Given that *P. aeruginosa* from wounds had higher *exoU* gene expression than isolates from burns and urine, and that *exoT* gene expression was comparatively consistent across all isolates, we deduce that this bacterium can adapt to a variety of pathogenic environments. Therefore, this study recommends conducting extensive research on *P. aeruginosa* isolated from various clinical cases and examining the impact of these genes on illness conditions.

Acknowledgments

I would like thank to patients who agreed to provide samples, also gave the personnel of Microbiology Labs of Burns Hospital and Al-Diwaniya Teaching Hospital.

Ethical approval

General ethics guidelines were followed when conducting the study. According to the mission facilitation letter No. 4277/27/7 dated October 2, 2024, the study topic was submitted to the appropriate committees and accepted. Before collecting the sample, the patient's consent was requested, taking into consideration their psychological and physical conditions.

Material and Data Availability

Upon request, data and results will be obtained from corresponding author.

Competing Interests

No interest conflict.

Funding

No funding.

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