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ABSTRACT

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FUR GENE EXPRESSION AND ITS RELATION WITH IRON-RESPONSIVE GENES IN PSEUDOMONAS AERUGINOSA ISOLATES FROM WOUNDS AND BURNS

Introduction. *Pseudomonas aeruginosa* is known as opportunistic and results in a variety of infections by the acquisition of iron from the host by iron-responsive genes, which are known as *Pvd* and *Pch*. Expression of these genes is controlled by a gene known as *Fur*, which is responsible for the regulation of genes mentioned above under iron availability conditions. This research aimed to study the relationship between *Fur* and iron-responsive genes in *Pseudomonas aeruginosa* under iron availability in order to use the iron in different concentrations to prevent and inhibit infections by *P. aeruginosa*.

Materials and methods. Forty isolates were collected and identified by culture and biochemical tests based on growth characteristics on ordinary culture media like blood, macConkey and cetrimide agar. Colonies of isolates appeared as large, irregular beta-hemolytic on blood agar, while they appeared colorless with a positive oxidase test on macConkey, and on a cetrimide agar, they appeared greenish. A biochemical test was achieved to confirm the pathogen is *P. aeruginosa*; results showed indol negative, citrate positive, urease negative, motility positive, and lastly, no fermentation of glucose and lactose. PCR was also used to confirm these isolates as *P. aeruginosa* by detection of *16SrRNA* as a reference gene. Different iron concentrations were prepared by equation c1v1=c2v2; then, the bacteria were cultured in sterile brain heart infusion with different iron concentrations and incubated for 24 h at 37 ℃. qPCR was performed on these isolates to assess the effect of *Fur* on gene expression of target genes under iron availability conditions.

Results. Results showed that iron-responsive genes were affected by the *Fur* gene in some isolates but not affected in others. We conclude from these results that the *Fur* gene controls the gene expression of iron-responsive genes under a universal environment, and other factors are also

required to regulate target genes, such as affinity, stability, and quality of the interaction between Fur and DNA and the structure of Fur boxes.

Conclusion. The prevalence of nosocomial infections caused by *P. aeruginosa* is increasing in Iraqi hospitals. Iron and *Fur* gene play an important role in the pathogenicity of bacteria because, under replete iron conditions, the *Fur* gene acts as an ap-oppressor and binds with iron, which acts as a co-pressor to repress expression of iron-responsive genes and vice versa to maintain the bacterial life and keeping iron homeostasis within bacteria.

Keywords: *Fur* gene, iron-responsive genes, *Pch and Pvd genes, Pseudomonas aeruginosa*.

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ABBREVIATIONS
Fur – ferric uptake regulator
Pvd – pyoverdin
Pch – pyochelin
PCR – polymerase chain reaction
qPCR – quantitative polymerase chain reaction

INTRODUCTION

Gram-negative bacteria (GNB) are among the world's most significant public health problems due to their high resistance to antibiotics [1]. The Pseudomonadaceae family is one of the largest families of Gm-negative bacteria. They are a group of self-sufficient organisms that are frequently seen as saprophytes in a variety of settings, including soil, plants, fresh water, and in conjunction with animals, where they can act as infectious agents that cause diseases in people, plants, and animals [2]. The organism under observation exhibits a remarkable degree of adaptability to various environmental factors, such as temperature variations, elevated concentrations of salts and dyes, mild antiseptics, and multiple commonly used antibiotics [3]. *P. aeruginosa* is gram-negative and does not undergo fermentation [4]. This bacteria has its own virulence factors, which play an important role in acute and chronic infections, such as pili, biofilm formation, lipopolysaccharide, pyocyanin, siderophores, and exotoxin [5]. According to the study conducted by [6], this rod-shaped, opportunistic gram-negative bacterium causes a variety of infections by producing many extracellular virulence factors, which are associated with extensive tissue damage, invasiveness, colonization, which enable bacteria to cause damage in the skin, eyes, ear, respiratory system. *P. aeruginosa* exhibits a variety of behaviors, including Quorum sensing, biofilm formation, and virulence factors, supported by intricate molecular pathways and genotypic alterations [7]. The identity of the organism is often determined by measuring its growth capacity at 42°C, observing the presence of characteristic colors, evaluating its colony form, and determining whether it is oxidase-positive. Although it can also perform anaerobic respiration by using various alternative electron acceptors, such as nitrate, it is aerobic and prefers to use oxygen as the final electron acceptor during aerobic respiration [8]. Treatment of infections caused by *P. aeruginos* is a big challenge as it exhibits resistance to a broad spectrum of antimicrobial agents [9]. *P. aeruginosa* is the third most prevalent etiology of urinary tract infections (UTIs) [10]. Nosocomial urinary tract infection (UTI) caused by *P. aeruginosa* is correlated with significant morbidity and death rates, and the development of bacteremia is a possible consequence [11]**.** Many different acute and chronic infectious diseases are caused by the bacterium. Generalized inflammation and sepsis are the signs of these infections, especially in patients with burns where the skin's host defenses are compromised, as well as in immune-compromised individuals with HIV or cancer infections [12]. Initially, burn wounds become colonized and infected with gram-positive bacteria, which are later replaced by gram-negative bacteria during the second week [13]. Cutaneous infections caused by *P. aeruginosa* are common in patients with wounds and burns. Skin infections typically result from compromised epithelial barriers, such as those that develop after trauma, burns, or surgical wounds, as well as those that develop around implants or other indwelling devices [14]. The bacterial

ability to form biofilms is a crucial component of their pathogenic activity, which poses a considerable challenge to the management of burn injuries. Moreover, it negatively impacts the survival rate of those who have experienced burn injuries [15]. Iron acquisition systems like siderophores in *p.aeruginosa* play a virulence factor that enables bacteria to survive within the host to initiate infection. Pyoverdine is the first type of siderophore that serves as the principal siderophore responsible for the acquisition of iron from the surrounding medium. These molecules exhibit scavenging properties and facilitate the transportation of iron to the intracellular compartment of the bacteria. Pyochelin is the second type of siderophore, which is classified as a salicylate-based siderophore with a comparatively reduced affinity for iron [16]. Gene transcription of these siderophores is controlled by the *Fur* gene, which represses gene transcription of these siderophores under iron repletion conditions, while under iron depletion conditions, *Fur* derepresses gene transcription of siderophores [17]**.**

MATERIALS AND METHODS

A total of 40 isolates of *P. aeruginosa* were collected from patients with burns and wounds who were admitted to Imam Ali Hospital, Alnumaan Teaching Hospital, AL-Kindy Hospital, and Ibn-Albaladi Hospital for women and children in Baghdad.

Isolation and identification

The diagnosis of bacterial isolates was validated by evaluating their capacity to grow on diagnostic media, specifically MacConkey agar, blood agar, and cetrimide. The study focused on examining the morphology, dimensions, and pigmentation of the developing colonies, as well as the alterations induced by these colonies on the growth media. Additionally, phenotypic traits were observed, and biochemical tests, including oxidase, catalase, and IMVc, were conducted.

Molecular method (DNA extraction)

This technique was conducted using the genomic DNA purification Kit supplemented by (Trans). A bacterial suspension was prepared by inoculating the isolates in Brain Heart Broth (BHB) media and incubating it at 37°C for 24 hours. The turbidity of the suspension was adjusted to approximately $1x10^9$ CFU/ml. Then, 1 ml of the suspension was transferred to a 1.5 ml Eppendorf tube and centrifuged at 14000 xg for 1 minute.

Conventional PCR for (*Fur, Pvd,* and *Pch*) genes

The detection of each gene was accomplished by the standard PCR process, which relied on specific primers. Table 1 shows specific primers. The PCR mixture is prepared by combining 12.5 µL of the master mix, 3 µL of the DNA sample, 1 µL of each primer at a concentration of 10 pmol/µL, and 7.5 µL of free-nuclease water. After completely homogenizing the contents of the PCR tubes and transferring them to the PCR apparatus, the resulting volume of the reaction mixture was 25 microliters. Subsequently, a volume of 5µl of the replicated gene was applied onto the previously prepared 1.5% agarose gel for electrophoresis.

Table 1 – Primers required in RNA extraction

Name of Primer	Sequence	Product size (bp)	Reference
16S rRNA	F-5`-GCCTCATGCCATCAGATGTGC-3` R-5`-GCAATATTCCCCACTGCTGCC-3`	158	Newly Designed
Fur	F-5`-ACCAGCCCGTATTCCTTCGTC-3` R-5`-CAGCGCAGGTAGTCGTTGAAC-3`	113	Newly Designed
Pvd	F-5`-ACCAGCCCGTATTCCTTCGTC-3` R-5`-CAGCGCAGGTAGTCGTTGAAC-3`	113	Newly Designed
Pch	F-5`-TTCCGAATCGCCTACCAGACC-3` R-5`-CAGCACGAAGGGATGGATCGT-3`	156	Newly Designed

Preparation of iron concentrations

Four iron concentrations (125 µM, 100 µM, 75 µM, 50 µM) were generated using the equation (C1V1 = C2V2). Three milliliters of brain heart broth were combined with three milliliters of each iron concentration in twenty-four sterile tubes. Subsequently, each tube was inoculated with a pure colony of bacteria that had been cultured overnight, and all tubes were cultured at 37 ℃ for 24 hours. Additionally, twenty-four sterile tubes containing only brain-heart broth were inoculated with the same colony and incubated overnight at 37 ℃ as a control. The growth of bacteria in each tube was monitored to determine the sub-minimum inhibitory concentration (sub-MIC). The growth of *Pseudomonas aeruginosa* in tubes with a concentration of 100 µM was analyzed using real-time PCR to determine the fold of expression of each gene compared to the control tubes.

RNA extraction

RNA extraction was conducted both before and after treatment of *P. aeruginosa* isolates with iron. The

extraction of total RNA was performed using TRIzol™ Reagent.

Primers used in this study

The source of all primers used in this study was Macrogen® (Korea). The name, sequence, and product size are given in Table 1.

Quantitative Real-time PCR Assay (QRT- PCR)

Amplification of mRNA fragments was conducted using the Qubit®. RT-qPCR System (Qubit®, USA) and the master amplification reaction are listed in Table 2 and Table 3. Multiple trials were conducted to optimize cDNA synthesis and annealing temperature.

Table 2 – Quantitative RT-PCR Reaction Mix

Component	20 ul Reaction
Luna Universal qPCR Master Mix	10 ul
Forward primer (10 µM)	1 ul
Reverse primer (10 µM)	1 ul
Template DNA	5 ul
Nuclease-free Water	3 ul
Total	20

Table 3 – Quantitative RT-PCR Reaction Mix

Cycle Step	Stage	Temperature	Time
1	Initial Denaturation	95 ℃	30 seconds
40	Denaturation Extension	95 ℃ 60 ℃	60 seconds 45seconds
1	Melt Curve	60–95 ℃	45 minutes

For this study, SYBR green, a fluorescent dye that attaches to and inserts itself between all forms of double-stranded DNA (including cDNA), was employed to measure the amount of DNA in real-time PCR. The *16srRNA* gene, as a reference gene, was used in the present experiment. Six isolates were treated with iron at a concentration of 100µM, and gene expression was assessed in two sets of *P. aeruginosa* isolates; one was treated with iron and the other as a control. The mRNA expression of the target genes was evaluated using a quantitative RT-PCR technique, and a comparison was made between the treated group and the untreated groups. RT-qPCR has emerged as the preferred method for measuring gene expression due to its simplicity, consistency, heightened sensitivity, and specificity. The approach of relative quantification is commonly employed to analyze differences in the expression of a target gene in relation to a reference gene. Molecular techniques such as PCR have been utilized in various areas of medical study and are recommended because they give accurate results and help in the rapid diagnosis of different diseases caused by pathogenic bacteria and genetic diseases [18-32].

RESULTS

EasyPure®Genomic DNA Extraction Kit (Transgene® China) was used to extract the DNA of 40 isolates from colonies classified as *P. aeruginosa*. Molecular detection was done for gene 16SrRNA in 40 isolates by conventional PCR technique. The results revealed that *16SrRNA* was located in 30 (75%) out of 40 *P. aeruginosa* isolates. Correspondingly, ten isolates were identified using the traditional method, such as P. aeruginosa, but they did not have this gene. Reverse transcription-quantitative real-time polymerase chain reaction (RT-q PCR) has become the method of choice for quantifying gene expression because of its simplicity, repeatability, high sensitivity, and specificity. Relative quantification is the most often used method for examining variations in a target gene's expression in comparison to a reference gene. Six *P. aeruginosa* isolates were utilized in a gene expression study to investigate the effect of the *Fur* gene on iron-responsive genes under iron availability conditions. The *Fur* gene is involved in the regulation of iron-responsive genes, such as *Pvd* and *Pch*, which play a role in the production of siderophores. Siderophores are crucial for the assimilation of iron from the host. The findings indicated that treatment with a sub-MIC (100µM) of iron led to an increase in *Fur* gene expression and up-regulation in four isolates and down-regulation in two isolates. This up-regulation, in turn, affected the gene expression of *Pvd* and *Pch* genes. The *Pvd* gene expression was down-regulated in only one isolate while up-regulated in the remaining isolates. The results of *Pch* gene expression showed that the gene was down-regulated in one isolate and up-regulated in all other isolates, as shown in Table 4.

DISCUSSION

Iron represents an essential element for pathogen proliferation; however, its accessibility is limited in the host organism because of its complex formation with proteins or heme molecules. Acquisition of iron is, therefore, considered a major challenge for host-invading microorganisms. To overcome the iron limitation, *P. aeruginosa* produces various pigments with siderophore properties, such as pyoverdine or pyochelin. Our results in this research were explained according to many studies by other researchers, so the results of *16SrRNA* were in agreement with [33] as they found that the results of *16S rRNA* were located in 50 (96.1%) out of 52 biochemically *P. aeruginosa* isolates. The absence of *16SrRNA* in some isolates may be due to

Table 4 – Fold of expression of Fur, Pvd and Pch pseudomonas aeruginosa. Isolates before and after treatment with iron at sub-mic

Target gene	*Fur*	*Pvd*	*Pch*
Isolate (1)	3.03	1.07	84.4
Isolate (2)	0.8	1.07	3.7
Isolate (3)	2.29	1.23	2.14
Isolate (4)	2.63	4.0	0.006
Isolate (5)	0.57	0.20	----------
Isolate (6)	6.06	5.2	5.6

the presence of other closely related species, so the conventional biochemical test was not as accurate as PCR assays; as mentioned in the study by [34], they use PCR as a simple, rapid and accurate assays for distinguishing between *P. aeruginosa* and other related pathogen genus; also, is the most specific in comparison to other diagnostic tests such as API 20, which yields a 70% as *P.aeruginosa*, while the *16S rRNA* test, yields a 90%. The Results of gene expression of *Fur*, *Pvd*, *Pch* in certain isolates were explained by many researchers as in [35, 36]; these studies indicate that the *Fur* gene, in the presence of a sufficient amount of iron, produces Fur protein, which acts as an apo-pressure and binds with iron as a co-pressure. This binding activates the Fur protein to be a transcriptional regulator, leading to the suppression of genes involved in iron acquisition and other virulence traits. Also, the results agree with the research [37], which demonstrated that the Fur protein, encoded by the *Fur* gene, serves as a prevalent transcriptional regulator. Using iron as a co-regulator, they attach to specific sequences (Fur boxes) found in a region called the upstream promoter of target genes. Previously, it was believed that this protein was solely responsible for regulating iron balance in most prokaryotes. The remaining isolates exhibited up-regulation in the gene expression of target genes when iron was available this due to several factors. The affinity, stability, and quality of the interaction between Fur and DNA are influenced by various factors, such as the structure of Fur boxes, the presence of divalent metal ions, the redox state of the Fur protein's cysteine and histidine residues, ionic interactions, and the involvement of effectors like heme. Any abnormalities or defects in these factors can result in inadequate Fur activity. The presence of sufficient fur protein products is not enough to repress gene expression because it may be inactive, so activity must be assessed [36].

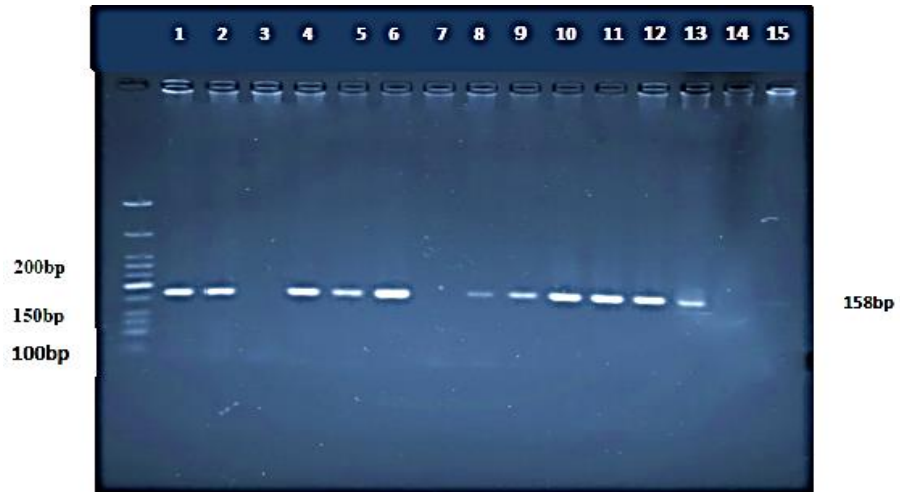

Figure 1 – Gel electrophoresis of amplified PCR product of *16SrRNA* gene (158b of *P. aeruginosa* bacterial samples were fractionated on 1.5% agarose gel electrophoresis. M: 25bp ladder marker)

CONCLUSION

The prevalence of nosocomial infections caused by *P. aeruginosa* is increasing in Iraqi hospitals. Iron and *Fur* gene play an important role in the pathogenicity of bacteria because, under replete iron conditions, the *Fur* gene acts as an ap-oppressor and binds with iron, which acts as a co-pressor to repress expression of iron-responsive genes and vice versa to maintain the bacterial life and keeping iron homeostasis within bacteria.

PROSPECTS FOR FUTURE RESEARCH

For future research, the relationship between the *Fur* gene and other virulence genes should be studied, and the amount and activity of Fur protein to be assessed.

AUTHOR CONTRIBUTIONS

All authors substantively contributed to the drafting of the initial and revised versions of this paper. They take full responsibility for the integrity of all aspects of the work.

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None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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