Pak. j. life soc. Sci. (2024), 22(1): 2262-2268 E-ISSN: 2221-7630;P-ISSN: 1727-4915

Pakistan Journal of Life and Social Sciences
www.pjlss.edu.pk

https://doi.org/10.57239/PJLSS-2024-22.1.00166

RESEARCH ARTICLE

Evaluation the effect of Cefotaxime on gene expression of Eno in Pseudomonas aeruginosa

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ARTICLE INFO

Received: May 28, 2024

Accepted: Jun 16, 2024

Keywords

Cefotaxime
Eno
Pseudomonas aeruginosa

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ABSTRACT

Pseudomonas aeruginosa is one of the most dangerous wound bacteria, and its resistance is being studied. Virulence is as important as removing the organism. Fifty wound samples from Baghdad hospitals were examined and identified by standard tests and VITEK-2. The genus was identified by detecting the particular gene Eno using the polymerase chain reaction (PCR) technology. Based on the findings of cultural and biochemical tests, only 12 isolates were recognized as Pseudomonas aeruginosa, the most common causal agent. Six isolates exhibiting the greatest antibiotic resistance had their minimum inhibitory concentrations (MICs) for Cefotaxime determined using the appropriate diffusion method. We calculated that the minimum inhibitory concentration (MIC) of cefotaxime was (1/8) µg/ml. All isolates had the Eno gene identified by PCR, demonstrating its significance in identifying the P. aeruginosa species and its innate molecular traits. The Eno gene expression was assessed by quantitative reverse transcriptase PCR. When Cefotaxime was applied to the wound samples, the gene expression fold value decreased as compared to the untreated isolates, according to the study.

INTRODUCTION

Opportunistic *Pseudomonas aeruginosa* causes recurrent infections that are difficult to treat because of its low resistance to antibiotics and the emergence of antibiotic resistance during therapy. Overuse of antibiotics is the main cause of the growing prevalence of multidrug resistance (MDR) **(1)**. Drug resistance in *P. aeruginosa* results from physiological and genetic causes including mutations in drug targets, loss of outer membrane proteins (porins), development of beta-lactamase enzymes, and multidrug efflux pumps. Third-generation cefotaxime is a recently produced semisynthetic cephalosporin **(2).** It works well against both Gram-positive and Gram-negative bacteria that proliferate in situations high in and low in oxygen. Furthermore, it usually works better against Gram-negative bacteria than the previous cephalosporin generations did **(3).** Although cefotaxime does work somewhat against *Pseudomonas aeruginosa*, the body of research does not yet support using it as the sole antibiotic for pseudomonal infections **(4).** Still, Cefotaxime has successfully cured infections brought on by other difficult bacteria, such Enterobacteriaceae that are resistant to multiple drugs **(5).** Like other cephalosporins, cefotaxime works well to treat patients with complicated urinary tract and lower respiratory tract infections, such pneumonia brought on by Gram-negative bacteria **(6).** High response rates have also been reported in patients with bacteremia of Gram-negative bacterias. *P. aeruginosa* deliberately controls the expression of a number of

virulence factors during infection to thwart the host's immune system and increase its antibiotic tolerance **(7,8).** *Eno* gene generates the enzyme Enolase. One essential enzyme in the glycolytic pathway—a metabolic route used by cells to break down glucose and generate ATP as energy—is enolase. At a critical point in glycolysis, enolase helps 2-phosphoglycerate (2-PG) become phosphoenolpyruvate (PEP) **(9).** The aims of this study are Evaluation the effect of Cefotaxime on gene expression of *Eno* in *Pseudomonas aeruginosa*

MATERIAL AND METHODS

Isolation and Identification of *Pseudomonas aeruginosa*

Between October 2022 and February 2023, fifty specimens were collected, comprising both males and females. The identification of *Pseudomonas aeruginosa* was determined based on its morphological characteristics on MacConkey agar, as well as its ability to produce oxidase and catalase enzymes, and its positive results in the methyl red, indole, urease, Voges-Proskauer, Kligler iron agar, citrate utilization, and motility tests. The VITEK 2 system validated the results.

Molecular study

The genomic DNA purification kit (Geneaid Extraction Kit) was used to separate and purify the DNA. Two µl of each extracted DNA sample was placed on the Nano-drop device's measurement lens. Before proceeding, the lens was cleaned by gently rubbing it with a cotton swab saturated in distilled water. Evaluate the concentration and purity of the extracted DNA sample by measuring its absorbance at wavelengths of 260 nm and 280 nm. The particular primers employed in the current investigation are enumerated in Table 1. Furthermore, Table 2 presents the specific PCR amplification methodology used for the *Eno* gene in this study.

Table (1): Primers used in this study

Primer	Sequence	Tm (°C)	GC (%)
Eno	**F:** 5'-GACGGTACTCCTAACAAAGGTAAA - 3'	68	70.46
	R: 5'- ATAGCTGTAAAGTGGGATTTCAAG- 3'	66	68.75
16srRNA	**F:**5′-CCACACTGGGACTGAGACAC -3′	57.5	60 %
	R:5′-CCACTCCCGCTAACGTTCTT -3′	57.2	55 %

Table (2): PCR amplification program for *Eno* used in this study

No.	Phase	Tm (°C)	Time	No. of cycle
1-	**Initial Denaturation**	**94°C**	**5 min.**	**1 cycle**
2-	**Denaturation -2**	**94°C**	**45sec**	**35 cycle**
3-	**Annealing**	**65°C**	**1min**	
4-	**Extension-1**	**72°C**	**45sec**	
5-	**Extension -2**	**72°C**	**10 min.**	**1 cycle**

The results were recorded via computerization. The DNA, primers, and PCR master mix were mixed. The PCR solution was produced in a total volume of 20µL, comprising 5µL of PCR Green master mix,

1μL of each primer, and 2μL of template DNA. The remaining volume was filled with sterile de-ionized distilled water, and the combination was subsequently agitated using a vortexing technique. The PCR procedure comprised 35 cycles. The process started with an initial denaturation phase at 94 ºC for 5 minutes. Therefore, the denaturation step at the same temperature for 45 seconds, an extension phase at 72 ºC for 45 seconds, and a concluding extension step at 72 ºC for 10 minutes. The annealing phase of Eno took place at a temperature of 65°C for 1 minute. The PCR findings were analyzed by subjecting them to 2% agarose gel electrophoresis in a 100 ml solution of 1x TBE buffer, followed by melting.

Minimum Inhibitory Concentrations (Antimicrobial Activity)

The agar well diffusion method is one of the most common approaches for determining an antibiotic's antibacterial efficacy. Isolates of *P. aeruginosa* were tested for their ability to fight off infections caused by the antibiotic cefotaxime, and the minimum inhibitory concentrations were found **(10).**

Gene expression analysis

The determination and computation of gene expression levels for one or more genes rely on the concentration of RNA/miRNA, which is converted into cDNA. The entire workflow encompasses the purification of total RNA, amplification of qPCR, and subsequent data processing. The RNA was extracted from the sample using the TRIzol™ Reagent technique. The primers utilized in this investigation are displayed in Table (1). The composition of the reaction mixture for one-step RT-PCR is provided in Table (3), while the Real-Time PCR procedure is outlined in Table (4).

Table (3): The components of the reaction mixture of One Step RT-PCR, final volume is 10µl

Component	20 µL (Final volume)	Final concentration
KAPA SYBR FAST qPCR Master Mix (2X) Universal	10 µL	2x
Forward primer	1 µL	0.2µM
Reverse primer	1 µL	0.2µM
Nuclease-free water	3	--
Template DNA Sample Volume	5	1pg-100ng

Table (4): Real Time PCR Program

Step	Temp. (°C)	Time	Cycle
Pre-denaturation	95 °C	05:00 min	Hold
Denaturation	95.0 °C	:00:30 sec	*40
Annealing	56.0 °C	:00:30 sec	
Extension	72.0 °C	:00:30 sec	

Gene Expression Calculation, Relative quantification

Folding =2-ΔΔCT

ΔΔCT =ΔCT Treated - ΔCT Control

ΔCT =CT gene - CT House Keeping gene

RESULTS

Collection of samples

Between October 2022 and February 2023, a total of fifty specimens were collected, representing both males and females. Twelve isolates were identified as *Pseudomonas aeruginosa* based on their morphology on MacConkey agar, Oxidase, Catalase, Methyl red, Indole, Urease, Vogeus Proskauer, Kligler iron, Citrate utilisation, and motility tests. The findings were validated using the VITEK 2 system.

Molecular study

The results of the research have identified a partial molecular characterization of the *Eno* gene in *Pseudomonas aeruginosa* bacteria using PCR technology. Out of 50 isolates, the result showed all (12, 17, 18) isolates of *Pseudomonas aeruginosa* carry the *Eno* gene **(fig 1).**

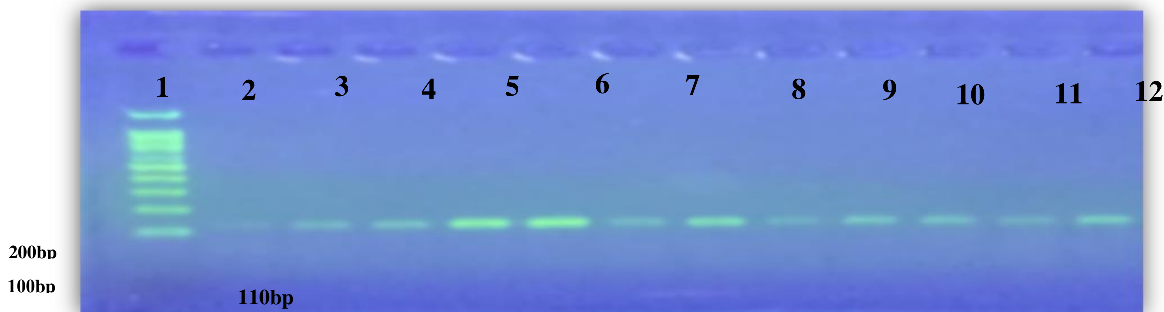

Fig (1) Gel electrophoresis of amplified *Eno* (110 bp) in *P*. aeruginosa isolates on agarose (2%), TBE buffer (1x), and 70 volts for 1 hr. stained with red safe L: DNA ladder (100 bp).

Minimum Inhibitory Concentrations (Antimicrobial Activity)

The results shown in Figure 2 are noteworthy because they indicate that Cefotaxime successfully hindered the growth of all the tested isolates at various dilutions (1/2, 1/4, 1/8, 1/16, and 1/32). The experiment's findings revealed that the minimum inhibitory concentration (MIC) of 1/8 successfully inhibited bacterial growth. The isolates were chosen based on their inhibition zone diameter and resistance to Cefotaxime. The findings indicated a statistically significant impact ($p < 0.05$) of Cefotaxime on *Pseudomonas aeruginosa* isolates (2, 3, 4, 8, 9, 10) compared to other isolates.

Fig (2): Antimicrobial Activity of Cefotaxime against *Pseudomonas aeruginosa*

Gene expression of *Eno* gene analysis by Quantitative Real Time PCR (qRT-PCR)

The gene expression level was determined by the behaviours of virulence factor Eno in *Pseudomonas aeruginosa* after being under-stressed with Cefotaxime. The current study investigated gene expression in eight isolates of *Pseudomonas aeruginosa* (numbers 2, 3, 4, 8, 9, 10). The RNA obtained from each isolate was extracted and used to assess the expression of the target virulence gene (*Eno*). The expression level of each gene was standardized and normalized using the housekeeping gene of *Pseudomonas aeruginosa* (16SrRNA). The Delta Ct values and gene expression folding are displayed in Table 5. The findings indicate that the expression of the *Eno* gene in *Pseudomonas aeruginosa* decreased following exposure to Cefotaxime-induced stress.

Table 5: Gene expression level of *Eno* in *Pseudomonas aeruginosa*

***P. aeruginosa* isolates**	**H.K.**	***Eno***	**ΔCt**	**ΔΔCt**	**folding**
2B	**21.9**	**22.6**	**0.7**	**0.00**	**1.00**
2A	**19.7**	**24.6**	**4.9**	**4.2**	**0.05**
3B	**22.8**	**23**	**0.2**	**0.00**	**1.00**
3A	**18**	**22**	**4**	**3.8**	**0.07**
4B	**24.3**	**25.8**	**1.5**	**0.00**	**1.00**
4A	**17**	**25**	**8**	**6.5**	**0.01**
8B	**23.8**	**26.4**	**2.6**	**0.00**	**1.00**
8A	**14**	**22**	**8**	**5.4**	**0.01**
9B	**23.9**	**33.6**	**9.7**	**0.00**	**1.00**
9A	**20**	**32.8**	**12.8**	**3.1**	**0.01**
10B	**20.4**	**20.9**	**0.5**	**0.00**	**1.00**
10A	**20**	**28**	**8**	**7.5**	**0.005**

***(B) isolates before treating with Cefotaxime**

DISCUSSION

Twelve isolates of P. aeruginosa were found when classical, biochemical, and VITEK 2 assays were applied to all 50 wound samples. Quicker decisions on therapy are made possible by the relatively short time it takes to get findings with the VITEK 2 technology. Particularly helpful in severe cases of P. aeruginosa infections, this is because a delay in identifying the organism and its susceptibility profile could have serious consequences. One study found that the VITEK 2 system could identify P. aeruginosa in an average of 9.2 hours; this could have serious consequences for patient treatment **(11).** As can be seen in Figure 1, all twelve P. aeruginosa isolates possess the Eno gene (1). An essential enzyme in Pseudomonas aeruginosa, a pathogenic bacterium well known for its adaptability and virulence, is encoded by this gene.The goal of this study is to demonstrate that the Eno gene is important for P. aeruginosa isolates and that it may influence bacterial pathogenicity and physiology. P. aeruginosa isolates have the multifunctional Eno gene, which plays an essential role in metabolism, pathogenicity, and the possible creation of vaccines. Bacterial physiology, host-pathogen interactions, and adaptation to the environment are all within the purview of this study. To better understand Pseudomonas aeruginosa and develop strategies to reduce its harmful effects, it is necessary to comprehend the roles played by the enolase enzyme and the Eno gene.The goal of this study is to determine, under controlled laboratory circumstances, the lowest dose of the antibiotic

Cefotaxime that will suppress the development of the specific bacterium Pseudomonas aeruginosa. Minimum Inhibitory Concentration (MIC) is the lowest concentration of Cefotaxime that, under controlled conditions, effectively stops the visible development of Pseudomonas aeruginosa **(12).** The Minimum Inhibitory Concentration (MIC) is a crucial metric for determining the sensitivity or resistance of Pseudomonas aeruginosa to Cefotaxime, a commonly prescribed antibiotic for numerous bacterial illnesses **(13).** The qRT-PCR analysis accurately measures Eno gene expression, allowing you to examine changes in gene expression across many samples or experimental settings. The present study demonstrated a downregulation of the Eno gene, which encodes the glycolysis-enzyme Enolase. Downregulation describes a decrease in the expression or activity of the relevant gene. Substantiating the relationship between Cefotaxime and the regulation of the Eno gene—which produces the glycolytic enzyme Enolase—requires additional data and comprehensive research in the scientific literature. Eukaryotic cells are the most common hosts for the gene enolase. Conversely, Cefotaxime is an antibiotic designed specially to target and impede the formation of bacterial cell walls. Enolase is a necessary part of the eukaryotic cells' glycolysis metabolic system. Regulatory alterations in the *Eno* gene can be brought about by a variety of physiological, developmental, or pathological factors. However, in most cases, these alterations are controlled by the cell's intrinsic regulatory systems **(14).** Mostly, cefotaxime is used to treat bacterial infections by focusing on certain pathways. The *Eno* gene is one of the eukaryotic genes whose direct control in host cells is yet unclear. Cefotaxime mostly targets bacterial cell processes; it usually does not directly control eukaryotic genes, like Eno, found in host cells. Usually, physiological or regulatory mechanisms inside eukaryotic cells rather than direct effect of cefotaxime are responsible for the decrease in expression of a specific gene, such *Eno* **(15).**

CONCLUSION

PCR is a dependable method for diagnosing *P. aeruginosa* strains obtained from various sources. By utilizing primers that target the Eno gene, this methodology yielded optimal outcomes when employed as a housekeeping gene in gene expression experiments, exhibiting low change across diverse sources. Identifying the target virulence gene *Eno*, which encodes for Enolase, an enzyme involved in glycolysis and carbon metabolism. All local isolates of *P. aeruginosa* were shown to be susceptible to the Cefotaxime antibiotic in the sensitivity test. Expose the resistant isolates to Cefotaxime to assess the gene expression of the *Eno* target gene. The expression of virulence genes decreased following exposure to Cefotaxime.

Acknowledgements: Mustansiriyah University/ Iraqi Centre for Cancer and Medical Genetic Research (www. uomustansiriyah.edu.iq) Baghdad-Iraq supported this work. Ethics statement

The Scientific Committee of the Department of Experimental Therapy, Iraqi Center for Cancer and Medical Genetics reviewed and approved the current studies.

Conflict of interest: (Non)

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