ISSN: 0067-2904

Investigation of Flagellum genes *FleN* and *FlgE* and Gene Expression of *FleN* Gene in *Pseudomonas Aeruginosa* Clinical Isolates

Zainab Waheed Kadim*, Hala Mouayed Radif
Department of Biology, College of Science, University of Baghdad, Iraq

Received: 21/5/2023 Accepted: 22/7/2023 Published: 30/8/2024

Abstract

The opportunistic multidrug resistance pathogen *Pseudomonas aeruginosa* has one or several flagella, and the numbers of these sophisticated machines are regulated by the flagellar regulator gene *FleN*. The flagellar hook gene *FlgE* is important for its synthesis, motility and tolerance to antibiotics. Bacteriahave resistance to antibiotics, especially to cephalosporin beta-lactam antibiotics. For the current study, 102 clinical specimens were collected and identified using routine laboratory tests and confirmed by Vitek-2 compact system. A total of 33 isolates of *P. aeruginosa* were identified. The antibiotic susceptibility test was done by the Vitek 2 Compact system. Flagellar gene detected by conventional PCR revealed that the *FleN* gene existed in 26 (78.8%), of which fifteen isolates were MDR. Whereas the *FlgE* gene existed in 20 (60.6%), of which fourteen isolates were MDR bacteria. The *FleN* gene expression in five isolates done by RT-qPCR revealed that four isolates were down regulated, and one isolate was up regulated in *FleN* gene expression when treated with ceftazidime at sub-MIC. While all isolates were downregulated when treated with amikacin at sub-MIC.

Keywords: *P. aeruginosa*, *FleN* gene, *FlgE* gene, Antibiotic resistance.

التحري عن جينات السوط *FleN* و*FlgE* والتعبير الجيني لجين *FleN* في الزائفة الزنجارية المعزولة سريرياً

زينب وحيد كاظم*, حلا مؤيد رديف
قسم علوم الحياة،كلية العلوم،جامعة بغداد،بغداد،العراق

الخلاصة

تمتلك بكتريا الزائفة الزنجارية الممرضة الانتهازية ذات المقاومة المتعددة للأدوية سوطاً واحداً او عدة اسواط، وعدد هذه آلات المتطورة ينظمها جين *FleN* المنظم لعدد الاسواط. جين خطاف السوط *FlgE* مهم لبناء السوط وللحركة ولتحمل المضادات الحيوية. البكتريا قاومت المضادات الحيوية وبشكل خاص مضادات البيتالاكتام السيفالوسبورينات. ١٠٢ عينه سريرية تم جمعها وشخصت بإستعمال الاختبارات المختبرية الروتينية، وتم تأكيد التشخيص بواسطة نظام الفايتك ٢ المدمج. في الدراسة الحالية، تم التعرف على ٣٣ عزلة زائفة زنجارية. اختبار الحساسية للمضادات الحيوية تم بوساطة نظام الفايتك ٢ المدمج. تضمنت النتائج المتعلقة بجينات السوط بواسطة تفاعل البلمرة المتسلسل التقليدي الاتي: *FleN* جين موجود في ٢٦ (٨.٧٨ ٪) منها ١٥ عزلة كانت متعددة المقاومة للمضادات الحيوية، بينما *FlgE* جين موجود في ٢٠ (٦٠.٦٪) منها

*Email: Zainab.Waheed1602a@sc.uobaghdad.edu.iq

١٤ عزلة كانت متعددة المقاومة للمضادات الحيوية. التعبير الجيني لجين *FleN* في خمس عزلات تم بواسطة النسخ الكمي العكسي لتفاعل البلمرة المتسلسل وكشف ان أربع عزلات تم تخفيض التنظيم فيها وعزلة واحدة تم رفع تنظيم التعبيرالجيني لجين *FleN* عندما عوملت مع تحت التركيز المثبط الادنى لمضاد السيفتازيديم. بينما جميع العزلات انخفض فيها تنظيم التعبير الجيني المذكور انفا عندما عوملت مع تحت التركيز المثبط الادنى لمضاد الأميكاسين.

1. Introduction

The Gram-negative bacillus *Pseudomonas aeruginosa* is an opportunistic [1, 2], facultative anaerobe [3], asporogenous bacteria that can appear individually or in short chains, and can move by having one polar flagellum or several flagella. It can also produce multiple virulence factors that are associated with both acute and chronic infections [4]. This pathogen lives in both biotic and abiotic environments and can be isolated from a variety of sites which include multiple nosocomial and life-threatening infections in patients with cystic fibrosis (CF patients), burns, injuries, urinary tract infections (UTI), ear and respiratory infections [5]. Due to its resistance to multiple drugs [7], it is regarded as a model for the emergence of antimicrobial resistance [6], making it more challenging to eradicate and increasing the incidence of persistent infections [6, 8].

Flagella are sophisticated biological macromolecular nanomachines, rotatable appendages on the surface of bacteria that allow them to swim in liquid media and swarm on semi-solid surfaces [9]. *Pseudomonas*' flagella are referred to as polar flagella because they are found at the cell's pole [10]. The biosynthesis and functions of flagella are controlled by about 50 genes [11] whereas the number of flagella and chemotactic motility in *P. aeruginosa* are controlled by the flagellar number regulator gene *FleN*. This gene encodes a protein that is made up of 280 amino acids and belongs to the P-loop NTPase family which is essential for preserving the number of flagella. Thus, this gene is critical for the establishment of the monotrichous phenotype. Maintaining the typical number of flagella and their distribution is crucial in motile bacterial species. This gene was found in significant amount in the majority of the cells which showed a complete stoppage of flagellar assembly.

Under a light microscope, the mutant *FleN* gene demonstrated tumbling, spinning and poor swimming abilities [12], indicating a deficiency in motility and chemotactic abilities [13]. In addition, the multiflagellate mutant outperforms its wild-type parent in terms of swarming. *FleN* in *P. aeruginosa* was the first gene to be partially identified as influencing both the number of flagellar filaments and motility in flagellated microbial species [12]. *FleN* inhibits *FleQ*, the primary global transcriptional regulator of flagellar genes [14] and biofilm genes [15, 16].

The bacterial flagellum is made up of a filament, a hook and a motor embedded in the cell envelope. The motor is made up of two components: the stator and the rotor; the last are made up of the switch complex and the basal body [17]. *Pseudomonas aeruginosa*'s flagellar hook, from which the flagellar filament protrudes, is comprised of 120 or so single molecules of flagellar hook protein E which is encoded by the flagellar hook gene *FlgE* [18]. The flagellar hook protein is known to be essential for flagellar synthesis and swimming motility [19].

FlgE modifications frequently cause structural abnormalities or deficiencies in a variety of bacterial species since it is involved in filament elongation or construction [20]. The *FlgE* knock-out mutant's biofilm cells exhibit increased tolerance to several antibiotics, whereas its planktonic cells exhibit resistance that is comparable to the wild type [21]. Also, *FlgE* mutant cells have been observed to have a higher susceptibility to permeabilization than the wild type

and get killed when exposed to innate immunity protein, the surfactant protein A. This finding indicates that *FlgE* has a significant role in bacterial resistance to host defense mechanisms [22]. However, it is worth noting that *FlgE* is a proinflammatory protein [18]. In some instances, the absence of *FlgE* has been detected in *P. aeruginosa* PAK cells grown in mucus [23]. The present study aimed to detect the relation between flagella genes and antibiotic resistance and study the *FleN* gene expression in MDR isolates under sub-MIC stress.

2. Materials and Methods

- **Samples Collection**

During the period from October 2022 to February 2023, patients were referred to three hospitals in Baghdad: Baghdad Medical City, Imam Ali (Jawader), and Martyr Sadr Hospitals, where one hundred and two different specimens were collected. The specimens were collected from wounds, burn wound swabs, mid-stream urine, ear swabs and sputum. Regarding swabs, they were taken with sterile cotton swabs and placed in a sterile tube containing transport media, then transferred to the laboratory.

- **Isolation and Identification of *Pseudomonas aeruginosa***

specimens were grown on MacConkey agar (Lab/England) and aerobically incubated for 24 hours at 37°C. The developed pale non-lactose fermenter colonies were selected, cultivated on Cetrimide agar (Himedia/ India), and also incubated in the same way. A single colony was inoculated on Cetrimide medium to carry out Gram-stain, oxidase, and catalase tests that are used for the identification of *P. aeruginosa*. The Vitek 2Compact system was tested using a Gram-negative (GN) card which included 48 biochemical examinations to confirm the bacterial isolate identification.

- **Antibiotic Susceptibility Test**

The antibiotic susceptibility test was performed by the Vitek 2-Compact system using the AST-N204 card for *P. aeruginosa*. Susceptibility, resistance, and MIC determinations and interpretations were automatically recorded for 33 clinical isolates.

- **DNA Extraction**

The DNA of 33 *P. aeruginosa* isolates was extracted via the EasyPure® Genomic DNA Kit (Transgene®, China). Qubit 4.0 (ThermoFisherScientific®, USA) was utilized to estimate the concentration of extracted DNA. The extracted DNA was stored at -20°C before use.

- **PCR in Silico and Primer Binding Site**

The reference genes (*FleN and FlgE*) were downloaded from GenBank as FASTA files. In the current investigation, Geneious Prime was downloaded using bioinformatics software for determining the proper annealing temperature and binding location for each pair of primers employed in this research. Additionally, the primers were designed using the exact same program and verified using a variety of online tools, primarily OligoAnalyzer from IDT (Integrated DNA Technology).

- **Detection of *FleN* and *FlgE* genes**

The examined genes were amplified using conventional PCR. The primers sequences are described in Table 1. A PCR mixture with a total volume of 25 microliters was prepared which consisted of 1 microliter of forward primer (10pmol /µl) (Macrogen, Korea), 1 microliter of reverse primer (10pmol /µl) (Macrogen, Korea), 12.5 microliters of Master Mix (New England Biolabs®,England), 3 microliters of template DNA, and 7.5 microliters of Nuclease-free water (New England Biolabs®,England). The solution was well mixed and vortexed to have homogeneous contents.

Table 1: Primers used in this study.

Primer Name		Sequence	Product Size (bp)	Annealing Temp. (°C)	Reference
FleN	F	5`-TTCGACGAAGAACTCCAGGTGCC-3`	175	58	Newly designed in this study
	R	5`-TGTAGGTGTCATCCCTTACGACGAGT-3`			
FlgE	F	5`-TGAACATCTGCGAGACGTCCGAG-3`	153	59	
	R	5`-ACGTCACCGGCAACAACATCG-3`			
16S rRNA	F	5`-ACTCCTACGGGAGGCAGCAGT-3`	198	50	[24]
	R	5`-TATTACCGCGGCTGCTGGC-3`			

- **PCR program**

The thermocycler was loaded with PCR reaction tubes filled with the mixture and DNA was amplified as in the conditions indicated in Table 2. The temperature and time of the PCR program were optimized by using gradient PCR.

Table 2: PCR amplification program for *FleN* and *FlgE* genes detection.

Step	Temperature (°C)	Time (Minute: Second)	Cycles
Initial Denaturation	94	5:00	1
Denaturation	94	00:30	30 X
Annealing	58^a,59^b	00:45	
Extension	72	00:45	
Final Extension	72	7:00	1

$FleN^a$ and $FlgE^b$

- **Gene Expression**

The effects of the most resistant antibiotics ceftazidime and amikacin on the gene expression of *FleN* were examined in five isolates with the highest levels of resistance and production of virulence factors. The bacterial cells were taken with and without ceftazidime or amikacin at sub-MIC. Two milliliters of Mueller Hinton broth, both with and without antibiotics at sub-MIC levels, were each added to 200 microliters of an overnight cultured diluted bacterial suspension standardized to 0.5 McFarland standard solution ($1.5\text{x}\,10^8$ CFU/ml) by using the Dens Check instrument [25].

RNA was extracted from *P. aeruginosa* using TRIzol™ Reagent by following the manufacturer's instructions. Qubit 4.0 was employed to estimate the purity and concentration of the extracted RNA. cDNA was synthesized by utilizing the Protoscript cDNA synthesis kit (New England Biolabs®, England), and the protocols were performed in a volume of 20µl. Each of the mixtures contained 10 µl of Protoscript reaction mix, 5 µlof the sample (purified RNA), 2 µlM-MuLV enzyme, 2 µl of random primer, and 1 µl of nuclease-free water. The real-time PCR program was then created utilizing the specified thermocycling technique (Table 3).

Table 3: q RT-PCR program

Cycle Step	Temperature(°C)	Time (Minute: Second)	Cycles
Initial Denaturation	95	1:00	1
Denaturation	95	00:15	
Annealing	58^a ,50^b	00:30	40-45
Extension	60	00:30	
Melt Curve	60-95	40 :00	1

$\boldsymbol{FleN^a}$ *and* $\boldsymbol{16SrRNA^b}$

- **Gene Expression Calculation**

The threshold cycle (Ct) was determined for each sample using the real-time cycler program. The housekeeping gene (*16S rRNA*) was used to standardize the expression data of the *FleN* gene. The q-PCR products were analyzed using the Livak technique [26].

Relative quantification

Fold of expression = $(2)^{-\Delta\Delta Ct}$

ΔΔCt = ΔCt treated (after treatment) - ΔCt control (before treatment)

ΔCt =Ct (for *FleN* gene)- Ct (for housekeeping gene)

- **Statistical Analysis**

For data entry and analysis, the Statistical Package for the Social Sciences (SPSS/ version 21, Software, San Diego, California, USA) was used. The mean, percentage and standard deviation of the mean were employed to summarize all the data. Continuous variables were defined as means ± standard deviation and categorical variables as absolute numbers and percentages (%). In this cross-sectional study, Chi square test was utilized to determine the significance level of the categorical variables. Cramér's V was used to detect the correlation between the antibiotic-resistant and flagellar genes. $P<0.05$ was regarded as statistically significant, while $P<0.01$ was regarded as extremely significant.

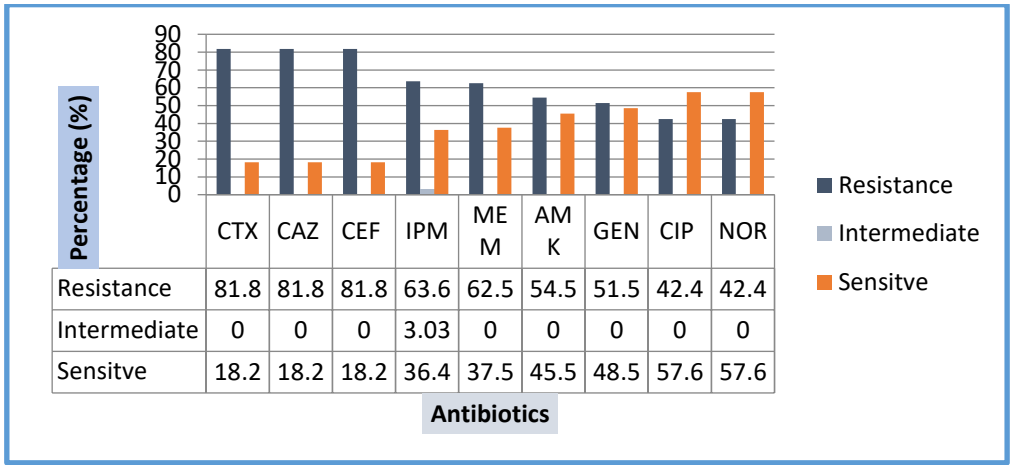
3. Results

From 102 bacterial specimens collected from patients referred to three hospitals in Baghdad, 33 (32.353%) were diagnosed as *P. aeruginosa* (Table 4), (54.55%) of isolates obtained from burn swabs, (18.18%) wound swabs, (12.12%) from urine specimens, (12.12%) ear swabs, and (3.3%) sputum specimens. On MacConkey agar, *P. aeruginosa* forms circular, mucoid, and smooth pale colonies that had a sweaty grape odor. While bacterial colonies showed greenish yellow on Cetrimide agar. Gram staining revealed that they were Gram-negative bacilli. In addition, catalase and oxidase were positive. The Vitek 2-test indicated that all 33 isolates were confirmed *P. aeruginosa*.

Table 4: Isolation percentage of bacteria according to specimen source

Specimen source	Positive n. (%) from 33 Isolates	Positive n. (%) from 102 Specimens
Burn	18 (54.55%)	(17.65 %)
Wound	6 (18.18%)	(5.88%)
Urine	4 (12.12%)	(3.92%)
Ear swab	4 (12.12%)	(3.92%)
Sputum	1 (3.03%)	(0.98%)
Total	33 (100%)	(32.35%)

All 33 *P. aeruginosa* isolates were examined via the VITEK 2-Compact system for antibiotic susceptibility to nine distinct drugs from four different antimicrobial categories. The findings were interpreted using the Clinical and Laboratory Standards Institute 2022 (CLSI) guidelines. Antibiotic susceptibility patterns toward Cephalosporin class showed high resistance compared with other antibiotics such as carbapenem, Aminoglycoside and Fluoroquinolones class. For instance 81.8% were resistance rates for cefotaxime, ceftazidime and cefepime. While the percentage of isolates resistant to carbapenems antibiotics imipenem and meropenem was 63.6% and 62.5% respectively, concerning aminoglycoside antibiotic resistance rates, *P. aeruginosa* isolates had a resistance rate of 54.5% and 51.5% for amikacin and gentamicin respectively. *P. aeruginosa* antibiotic susceptibility patterns toward Fluoroquinolones class showed the lowest resistance, such as 42.4% resistance rates for ciprofloxacin and norfloxacin.

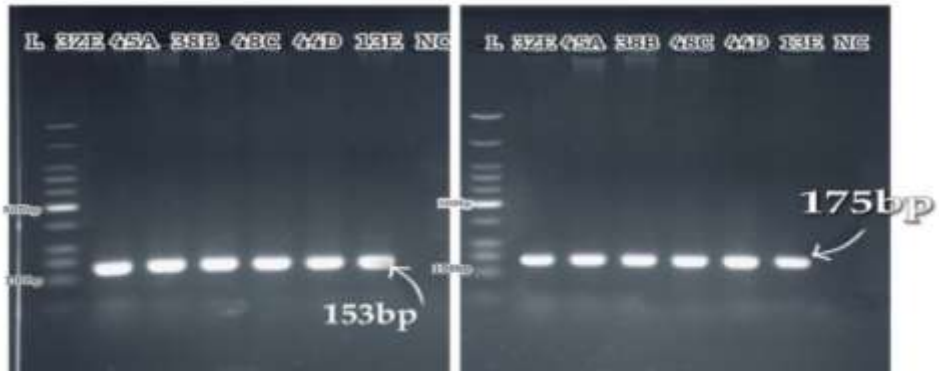


	CTX	CAZ	CEF	IPM	MEM	AMK	GEN	CIP	NOR
Resistance	81.8	81.8	81.8	63.6	62.5	54.5	51.5	42.4	42.4
Intermediate	0	0	0	3.03	0	0	0	0	0
Sensitve	18.2	18.2	18.2	36.4	37.5	45.5	48.5	57.6	57.6

Figure 1: Antibiotic resistance frequency of *P. aeruginosa* isolates, Cefotaxime (CTX), Ceftazidime (CAZ), Cefepime (CEF), Imipenem (IPM), Meropenem (MEM), Amikacin (AMK), Gentamicin (GEN), Ciprofloxacin (CIP), and Norfloxacin (NOR).\

The results of conventional PCR for the *P. aeruginosa* flagellar genes *FleN* and *FlgE*, which were detected in 33 isolates, indicated that the frequency of the present *FleN* gene was 26 (78.8%), of which fifteen isolates were MDR, whereas the *FlgE* gene was 20 (60.6%), of which fourteen isolates were MDR bacteria.

The isolates' PCR products were displayed on an agarose gel, with the bands compared to a DNA ladder size range of 100–1517 bp as shown in Figure (2).



(a) (b)

Figure 2: Visualization results of the amplification of (a) *FlgE* and (b) *FleN* genes in *P. aeruginosa* isolates were fractionated on 2% agarose gel electrophoresis stained with red safe. L: 100 bp ladder marker. Lanes 3ZE-13E resemble 153 bp for (a) and 175 bp for (b) PCR products.

The statistical correlation between antibiotic resistance and the presence of the *FleN* and *FlgE* genes in *P. aeruginosa* isolates is illustrated in Table 5.

Table 5: Correlation between antibiotic resistant isolate and presence of *FleN* and *FlgE* gene

		Antibiotic Susceptibility		*P*- value
		MDR Isolate	Sensitive Isolate	
FleN	Positive	15 (83.3%)	11 (73.3%)	0.088 NS
	Negative	3 (16.7%)	4 (26.7%)	
FlgE	Positive	14 (77.8%)	6 (40.0%)	<0.001**
	Negative	4 (22.2%)	9 (60.0%)	

**Correlation is highly significant at the 0.01 level (1-tailed) and non-significant at 0.088

The expression of the *FleN* gene in five *P. aeruginosa* isolates was studied using the RT-qPCR technique. Based on the findings presented in Table 6, four of these five isolates were downregulated when treated with Ceftazidime at sub-MIC, and one isolate was upregulated.

Table 6: Effects of ceftazidime on gene expression

Isolate Code	MIC (µg/ml)	Sub MIC (µg/ml)	Before Treatment with Ceftazidime			After Treatment with ceftazidime			ΔΔCt	Fold of Expression
			Ct 1 of *16S rRNA*	Ct 1of *FleN* gene	ΔCt	Ct 2 of *16S rRNA*	Ct 2 of *FleN* gene	ΔCt		
45 A	128	64	12.73	14.62	1.89	9.87	17.47	7.6	5.71	**0.019103754▼**
38 B	128	64	13.68	12.24	-1.44	10.44	13.51	3.07	4.51	**0.043889▼**
48 C	128	64	15.03	16.69	1.66	10.6	15.9	5.3	3.64	**0.080214▼**
44D	128	64	9.1	16.17	7.07	9.85	15.37	5.52	-1.55	**2.928171▲**
13 E	64	32	15.03	14.18	-0.85	12.82	14.42	1.6	2.45	**0.183011▼**

*Red color indicates down regulation of gene expression, black color indicates up regulation of gene expression

While the expression of the *FleN* gene in the same isolates was downregulated when treated with Amikacin at sub-MIC (Table 7).

Table 7: Effects of amikacin on gene expression

Isolate Code	MIC (µg/ml)	Sub-MIC (µg/ml)	Before Treatment with Amikacin			After Treatment with Amikacin			ΔΔCt	Fold of Expression
			Ct 1 of *16S rRNA*	Ct 1of *FleN* gene	ΔCt	Ct 3 of *16S rRNA*	Ct 3of *FleN* gene	ΔCt		
45 A	64	32	12.73	14.62	1.89	13.31	28.03	14.72	12.83	**0.000137336▼**
38 B	32	16	13.68	12.24	-1.44	13.06	29.36	16.3	17.74	**4.56802E-06▼**
48 C	32	16	15.03	16.69	1.66	13.28	27.75	14.47	12.81	**0.000139253▼**
44D	64	32	9.1	16.17	7.07	13.44	28.83	15.39	8.32	**0.003129179▼**
13 E	64	32	15.03	14.18	-0.85	6.96	17.66	10.7	11.55	**0.000333506▼**

*Red color indicates down regulation of gene expression, black color indicates up regulation of gene expression.

4. Discussion

Out of 102 specimens collected from various clinical specimens, only 33 isolates were identified as *P. aeruginosa*. The highest percentage of isolation was from burns (42%), followed by 15% from wound swabs, 12% from urine specimens, 12% from ear swabs, and 9% from respiratory system specimens (sputum and bronchial washes) [27]. This result agrees those of the study by Rashad *et al*.'s **[27]**. The high prevalence of *P. aeruginosa* isolates was available in burns due to the fact that burn patients are more susceptible to opportunistic bacterial colonization than others [28]. The bacterial colonies on MacConkey agar appeared pale yellow since they had not fermented lactose. *Pseudomonas* were grown on cetrimide agar, a selective medium for this bacterium since *P. aeruginosa* can withstand cetrimide material, which is toxic to other bacteria [29]. The cultural characteristics and microscopic examination results agree with those reported by AL-Rubaye*et al*. [30].

The Vitek 2 test demonstrated that 33 isolates had up to a 97% probability of *P. aeruginosa*. It was previously demonstrated that the Vitek 2 diagnostic approach is faster and less labor-intensive than traditional biochemical methods[31]. The outcome of this study aligned with previous research by Z. M. Jaafar *et al.* [32]**.**

The findings of the antibiotic susceptibility test in the present study showed resistance to ceftazidime which matches the results of Khosravi and Mihanion's research in Ahawaz, Iran (81%) [33], and to cefotaxime which is identical to the results of the Iraqi study by Alkhulaifi and Mohammed (81%) [34]. The result of this study was in contrast to Sweedan's findings who reported that *P. aeruginosa* isolates had the highest sensitivity rate against cefotaxime, thus showing a high considered difference [35]. While cefepime resistance was close with the results of one of the aforementioned local study, which was (80.3%).

The elevated resistance of *P. aeruginosa* isolates to cephalosporin category could be attributed to the breaking down of the β-lactam ring which interferes with the structure of antibiotics and inactivates them through the formation of β-lactamase enzymes. This is accomplished through plasmid-mediated mechanisms or by lowering membrane permeability to antibacterial drugs [36].

The results of resistance to carbapenems antibiotics agreed with those of Shilba *et al.* [37] who observed resistance to imipenem and meropenem of being 58.33% and 66.67% respectively .The results, however, disagree with Al-Shwaikh [38] who discovered that all *P. aeruginosa* isolates were 100% sensitive to imipenem. Also, the current study findings are not compatible with those of AL-Khazali [39] who discovered that *P. aeruginosa* isolated has low resistance to imipenem (42.1%), and that differs from Gailiene *et al*. [40].

Carbapenem resistance mechanisms have appeared due to the pressure of carbapenem used in clinical settings, and they can be classified as enzymatic, mediated by carbapenemases (beta-lactamases that hydrolyze carbapenems and other beta-lactams), or non-enzymatic. Carbapenem resistance, on the other hand, occurs commonly as a result of the coexistence of many mechanisms [41].

This study showed that *P. aeruginosa* isolates had a moderate resistance level against aminoglycosides, conflicting with the redaction resistance rate obtained by Al-Shamari and Al-Khteeb [42] who observed that the level of bacteria resistance to amikacin and gentamicin was 25% and 31.3% respectively.

The modification of target binding sites on the 16S ribosomal RNA by RNA methyltransferases can confer resistance to aminoglycosides, except for streptomycin. This

pathway is capable of producing significant levels of resistance to all other types of aminoglycosides [43].

The results revealed that although *P. aeruginosa* isolates have minimal resistance to Fluoroquinolone antibiotics which are still higher than the resistance rates of ciprofloxacin (20%) and norfloxacin (16.7%), according to Fadhel Abb*et al*.'s results [44].
Resistance to fluoroquinolones emerged in *P. aeruginosa* through a variety of mechanisms, including mutations in the genes encoding bacterial DNA topoisomerase II and topoisomerase IV which is a primary cause of fluoroquinolone resistance in *P. aeruginosa* isolates. At the same time, overexpression of active efflux systems can diminish membrane permeability. Additionally, it has been discovered that plasmids carrying the extended-spectrum beta-lactamase (ESBL) gene can also carry the quinolone resistance gene which may explain the high level of quinolone resistance in ESBL-producing strains [45].

The results of conventional PCR for the *FleN* gene indicated that gene percentage in clinically isolated specimens was 78.8%. Whilst the result showed that the *FlgE* gene percentage in clinically isolated specimens was 60.6%. Although many studies have been done about the *FleN* and *FlgE* genes in *P. aeruginosa*, no single study has used conventional PCR to detect the frequency of these genes in clinical isolates.
The statistical correlation between antibiotic resistance and the presence of the *FleN* gene in *P. aeruginosa* isolates revealed a non-significant correlation. In *P. aeruginosa* deletion of *FleN* is associated with the formation of multiple polar flagella. The multiflagellate mutant has a better capacity for swarming [12]. Swarming is associated with increased antibiotic resistance to different antibiotics [46].
Whereas a highly significant relationship is present between antibiotic resistance and the presence of the *FlgE* gene. Previous research has shown for the first time that the flagellar hook protein *FlgE* is involved in improved biofilm resistance of *P. aeruginosa* to many antibiotics.
The results highlighted in Table 6 reveal that four isolates were downregulated in *FleN* gene expression when treated with ceftazidime at sub-MIC, whereas one isolate was upregulated. There are insufficient studies to support treatment with ceftazidime at sub-MIC. While the treatment with amikacin in Table 7 downregulated the gene expression of the *FleN* gene in all five isolates. The current study also agrees with a previous study that treated *P. aeruginosa* with amikacin and other antibiotics at MIC and MBC and found that the gene expression of the *FleN* gene was downregulated in all isolates [47]. Mutations in the promoter region occur under antibiotic stress which affects the expression rates of target genes [48]. Significantly reduced expression of *FleN* has crucial regulatory effects as a flagellar regulon regulator. Significantly reduced expression of *FleN* has crucial regulatory effects as a flagellar regulon regulator. The results indicated a reduction in flagella-related motility. Flagellated cell decrease may potentially impair *P. aeruginosa* virulence via its association with invasive virulence [49].

5. Conclusions

Our study concluded that flagellar genes (*FleN* and *FlgE*) were present in most clinically isolated *P. aeruginosa* in different percentages. The statistical analysis revealed a significant correlation between antibiotic resistance and the *FlgE* gene and vice versa with the *FleN* gene. The *FleN* gene expression was downregulated by the sub-MIC of ceftazidime and amikacin stress in all isolates, except one isolate that was upregulated via the ceftazidime sub-MIC.

References

[1] T. Wagner, G. Soong, S. Sokol, L. Saiman, and A. Prince, "Effects of azithromycin on clinical isolates of Pseudomonas aeruginosa from cystic fibrosis patients," *Chest*, vol. 128, no. 2, pp. 912-919, 2005.

[2] J. Govan, "Pseudomonas aeruginosa and non fermenters, Medical Microbiology," *Churchill Livingtone Elsevier*, 2007.

[3] P. Liang *et al.*, "The aerobic respiratory chain of Pseudomonas aeruginosa cultured in artificial urine media: Role of NQR and terminal oxidases," *PLoS One*, vol. 15, no. 4, p. e0231965, 2020.

[4] S. García-Reyes, G. Soberón-Chávez, and M. Cocotl-Yanez, "The third quorum-sensing system of Pseudomonas aeruginosa: Pseudomonas quinolone signal and the enigmatic PqsE protein," *Journal of Medical Microbiology*, vol. 69, no. 1, pp. 25-34, 2020.

[5] M. W. Azam and A. U. Khan, "Updates on the pathogenicity status of Pseudomonas aeruginosa," *Drug discovery today*, vol. 24, no. 1, pp. 350-359, 2019.

[6] Z. Pang, R. Raudonis, B. R. Glick, T.-J. Lin, and Z. Cheng, "Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies," *Biotechnology advances*, vol. 37, no. 1, pp. 177-192, 2019.

[7] N. N. Yaseen and D. A. Ahmed, "Detection of mexB Multidrug Efflux Gene in Some Local Isolates of Pseudomonas aeruginosa," *Iraqi Journal of Science*, pp. 111-118, 2023.

[8] A. D. Khosravi, M. Motahar, and E. Abbasi Montazeri, "The frequency of class1 and 2 integrons in Pseudomonas aeruginosa strains isolated from burn patients in a burn center of Ahvaz, Iran," *PloS one*, vol. 12, no. 8, p. e0183061, 2017.

[9] M. Bouteiller *et al.*, "Pseudomonas flagella: generalities and specificities," *International Journal of Molecular Sciences*, vol. 22, no. 7, p. 3337, 2021.

[10] S. Kojima, H. Terashima, and M. Homma, "Regulation of the single polar flagellar biogenesis," *Biomolecules*, vol. 10, no. 4, p. 533, 2020.

[11] R. Macnab, "Flagella and motility," *Escherichia coli and Salmonella*, 1996.

[12] N. Dasgupta, S. K. Arora, and R. Ramphal, "fleN, a gene that regulates flagellar number in Pseudomonas aeruginosa," *Journal of bacteriology*, vol. 182, no. 2, pp. 357-364, 2000.

[13] N. Dasgupta and R. Ramphal, "Interaction of the antiactivator FleN with the transcriptional activator FleQ regulates flagellar number in Pseudomonas aeruginosa," *Journal of bacteriology*, vol. 183, no. 22, pp. 6636-6644, 2001.

[14] D. Jain, "Cloning, expression, purification, crystallization and initial crystallographic analysis of FleN from Pseudomonas aeruginosa," *Acta Crystallographica Section F: Structural Biology Communications*, vol. 72, no. 2, pp. 135-138, 2016.

[15] B. Y. Matsuyama, P. V. Krasteva, C. Baraquet, C. S. Harwood, H. Sondermann, and M. V. Navarro, "Mechanistic insights into c-di-GMP–dependent control of the biofilm regulator FleQ from Pseudomonas aeruginosa," *Proceedings of the National Academy of Sciences*, vol. 113, no. 2, pp. E209-E218, 2016.

[16] P. Banerjee and D. Jain, "ATP-induced structural remodeling in the antiactivator FleN enables formation of the functional dimeric form," *Structure*, vol. 25, no. 2, pp. 243-252, 2017.

[17] X.-Y. Zhuang and C.-J. Lo, "Construction and loss of bacterial flagellar filaments," *Biomolecules*, vol. 10, no. 11, p. 1528, 2020.

[18] Y. Shen *et al.*, "Flagellar hooks and hook protein FlgE participate in host microbe interactions at immunological level," *Scientific reports*, vol. 7, no. 1, p. 1433, 2017.

[19] I. Duchesne, T. Galstian, and S. Rainville, "Transient locking of the hook procures enhanced motility to flagellated bacteria," *Scientific reports*, vol. 7, no. 1, pp. 1-9, 2017.

[20] Y. Qin, G. Lin, W. Chen, X. Xu, and Q. Yan, "Flagellar motility is necessary for Aeromonas hydrophila adhesion," *Microbial pathogenesis*, vol. 98, pp. 160-166, 2016.

[21] J. D. Valentin *et al.*, "Role of the flagellar hook in the structural development and antibiotic tolerance of Pseudomonas aeruginosa biofilms," *The ISME Journal*, vol. 16, no. 4, pp. 1176-1186, 2022.

[22] S. Zhang, F. X. McCormack, R. C. Levesque, G. A. O'Toole, and G. W. Lau, "The flagellum of Pseudomonas aeruginosa is required for resistance to clearance by surfactant protein A," *PloS one*, vol. 2, no. 6, p. e564, 2007.

[23] J. Jyot, A. Sonawane, W. Wu, and R. Ramphal, "Genetic mechanisms involved in the repression of flagellar assembly by Pseudomonas aeruginosa in human mucus," *Molecular microbiology,* vol. 63, no. 4, pp. 1026-1038, 2007.

[24] R. J. Clifford *et al.*, "Detection of bacterial 16S rRNA and identification of four clinically important bacteria by real-time PCR," (in eng), *PLoS One,* vol. 7, no. 11, p. e48558, 2012, doi: 10.1371/journal.pone.0048558.

[25] C. H. Teh, W. A. Nazni, A. H. Nurulhusna, A. Norazah, and H. L. Lee, "Determination of antibacterial activity and minimum inhibitory concentration of larval extract of fly via resazurin-based turbidometric assay," *BMC microbiology,* vol. 17, no. 1, pp. 1-8, 2017.

[26] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method," *methods,* vol. 25, no. 4, pp. 402-408, 2001.

[27] F. F. Rashad, S. S. Obaid, and N. A. Al-kadhi, "Association of Multidrug Resistance With Biofilm Formation in Pseudomonas Aeruginosa Isolated from Clinical Samples in Kirkuk City," *NTU Journal of Pure Sciences,* vol. 1, no. 4, pp. 10-19, 2022.

[28] N. A. Chaudhary *et al.*, "Epidemiology, bacteriological profile, and antibiotic sensitivity pattern of burn wounds in the burn unit of a tertiary care hospital," *Cureus,* vol. 11, no. 6, 2019.

[29] B. A. Forbes, D. F. Sahm, and A. S. Weissfeld, *Diagnostic microbiology*. Mosby St Louis, 2007.

[30] M. R. S. AL-Rubaye, T. K. Mohammed, and H. N. Abdullah, "Isolation and Diagnosis of Multi Drug Resistance Pseudomonas Aeruginosa from Wound and Burnpatients in Baghdad City," *Indian Journal of Forensic Medicine & Toxicology,* vol. 14, no. 3, pp. 2431-2437, 2020.

[31] H. P. Dwivedi *et al.*, "Multicenter Clinical Evaluation of Vitek 2 Meropenem-Vaborbactam for Susceptibility Testing of Enterobacterales and Pseudomonas aeruginosa," *Journal of Clinical Microbiology,* vol. 60, no. 1, pp. e01610-21, 2022.

[32] Z. M. Jaafar, M. A. Dhahi, A. K. H. Abd, and S. M. Jaafar, "Molecular identification and antibiotics resistance genes profile of Pseudomonas aeruginosa isolated from Iraqi patients," *African Journal of Microbiology Research,* vol. 8, no. 21, pp. 2183-2192, 2014.

[33] A. D. Khosravi and F. Mihani, "Detection of metallo-β-lactamase–producing Pseudomonas aeruginosa strains isolated from burn patients in Ahwaz, Iran," *Diagnostic microbiology and infectious disease,* vol. 60, no. 1, pp. 125-128, 2008.

[34] Z. M. Alkhulaifi and K. A. Mohammed, "The Prevalence of Cephalosporins resistance in Pseudomonas aeruginosa isolated from clinical specimens in Basra, Iraq," *University of Thi-Qar Journal of Science,* vol. 10, no. 1 (SI), 2023.

[35] E. G. Sweedan, "The Antimicrobial Effects of Alcoholic Leaves Extract of Salvia Officinalis Against Multidrug Resistant Pseudomonas Aeruginosa," *Iraqi Journal of Science,* pp. 441-448, 2021.

[36] R. Al-Falahy, "Bacteriological study on some isolates of Ps. aeruginosa resistant to antibiotics and study plasmid content," M. Sc. thesis, Al-Mustansiryia University, 2000.

[37] A. A. Shilba, R. H. Al-Azzawi, and S. J. Al-Awadi, "Dissemination of Carbapenem resistant Pseudomonas aeruginosa among burn patients in Karbala Province\Iraq," *Iraqi J Sci,* vol. 56, pp. 1850-7, 2015.

[38] R. Al-Shwaikh, "Production and characterization of protease from Pseudomonas aeruginosa isolated from some clinical cases and its relation with some antibiotic agents," *PhD. desseration Biology/Microbiology/College of Science/AL-Mustansiryia University,* 2006.

[39] K. Al-khazali, "Resistance of Pseudomonas aeruginosa and Staphylococcus aureus isolated from burns and wounds infections to antibiotics and some disinfectants," M. Sc. Thesis. College of Science, AL-Mustansriyia University, Iraq, 2009.

[40] G. Gailienė, A. Pavilonis, and V. Kareivienė, "The peculiarities of Pseudomonas aeruginosa resistance to antibiotics and prevalence of serogroups," *Medicina,* vol. 43, no. 1, p. 36, 2007.

[41] G. Meletis, M. Exindari, N. Vavatsi, D. Sofianou, and E. Diza, "Mechanisms responsible for the emergence of carbapenem resistance in Pseudomonas aeruginosa," *Hippokratia,* vol. 16, no. 4, p. 303, 2012.

[42] R. K. Al-Shamari and S. N. Al-Khteeb, "Molecular Characterization Aminoglycosids Resistance Pseudomonas aeruginosa," *Iraqi Journal of Science,* pp. 1150-1157, 2016.

[43] J. N. O'Donnell, M. R. Bidell, and T. P. Lodise, "Approach to the Treatment of Patients with Serious Multidrug-Resistant Pseudomonas aeruginosa Infections," *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy,* vol. 40, no. 9, pp. 952-969, 2020.

[44] S. Fadhel Abb, A. S. Al-Khafaji, and H. M Radif, "Investigation of plasmid-associated fluoroquinolone resistance in nosocomial Pseudomonas aeruginosa isolated from infected burn wounds," *Journal of Biological Sciences*, vol. 18, no. 8, pp. 514-519, 2018.

[45] J. Ruiz, "Transferable mechanisms of quinolone resistance from 1998 onward," *Clinical microbiology reviews*, vol. 32, no. 4, pp. e00007-19, 2019.

[46] S. Rütschlin and T. Böttcher, "Inhibitors of bacterial swarming behavior," *Chemistry–A European Journal*, vol. 26, no. 5, pp. 964-979, 2020.

[47] M. Abu-Sini, M. A. Al-Kafaween, R. M. Al-Groom, and A. B. M. Hilmi, "Comparative in vitro activity of various antibiotic against planktonic and biofilm and the gene expression profile in Pseudomonas aeruginosa," *AIMS microbiology*, vol. 9, no. 2, p. 313, 2023.

[48] N. Händel, J. M. Schuurmans, Y. Feng, S. Brul, and B. H. ter Kuile, "Interaction between mutations and regulation of gene expression during development of de novo antibiotic resistance," *Antimicrobial agents and chemotherapy*, vol. 58, no. 8, pp. 4371-4379, 2014.

[49] M. A. Al-Kafaween, M. H. Abu Bakar, H. A. N. Al-Jamal, N. A. Elsahoryi, N. B. Jaffar, and M. K. Zahri, "Pseudomonas aeruginosa and Streptococcus pyogenes exposed to Malaysian trigona honey in vitro demonstrated downregulation of virulence factor," *Iranian Journal of Biotechnology*, vol. 18, no. 4, pp. 115-123, 2020.