Original Article

Investigation of the Flagellar Genes *FleN* and *FlgE* in Clinical *Pseudomonas aeruginosa* Isolates

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Abstract

Background: *Pseudomonas aeruginosa* is one of the most prevalent etiological agents that cause mortality and morbidity in hospitalized patients. Its sophisticated macromolecular appendage, known as the flagellum, plays important roles in bacterial movement, the infection cycle, and antibiotic resistance. *P. aeruginosa* possesses one or more polar flagella, and the number of these macromolecules is determined by the *FleN* gene, which also affects other flagellar regulatory genes, bacterial motility, and, consequently, virulence. Meanwhile, the *FlgE* gene encodes the hook portion of flagella and has an impact on the synthesis of intact flagella and their motility. Therefore, these genes play pivotal roles in *P. aeruginosa* virulence, and confirming their presence in clinical isolates is of utmost importance for assessing the intensity of virulence and pathogenicity. **Objectives:** The current study aimed to detect the presence of the flagellar genes *FleN* and *FlgE* in clinical *P. aeruginosa* isolates, and this study is considered the first of its kind to explore that. **Materials and Methods:** About 102 specimens from various clinical sources, such as burn, mid-stream urine, wound, ear, and sputum, were collected from patients referred to Baghdad Medical City, Imam Ali, and Shahid Al-Sadr Hospitals in Baghdad, Iraq. All isolates were identified based on phenotypic characteristics, biochemical tests, and confirmation by the Vitek 2 compact system. Conventional PCR was employed to detect flagellar genes. **Results:** Out of 102 specimens, 33 *P. aeruginosa* isolates were identified. The finding of conventional PCR indicated the presence of the *FleN* gene in 26 (78.8%) of these isolates, with the highest percentage found in burn isolates, followed by wound, urine, ear, and sputum isolates. Meanwhile, the *FlgE* gene was detected in 20 (60.6%) of the isolates, with the highest percentage observed in burn isolates, followed by wound and ear isolates, and the lowest percentage in urine and sputum isolates. **Conclusion:** The pathogenic bacterium *P. aeruginosa* is widely distributed in clinical sites, and the majority of them carry the flagellar genes *FleN* and *FlgE*.

Keywords: flagellum, *FleN* gene, *FlgE* gene and flagellar genes prevalence in clinical source, *P. aeruginosa*

Introduction

The pathogenic *Pseudomonas aeruginosa* bacterium is a facultative anaerobe Gram-negative motile rods.[1] It can move by having one polar flagellum or several flagella.[2] The bacterial flagellum, a filamentous cellular component, serves as a crucial factor for bacterial motility.[3] Additionally, it fulfills pivotal functions in various aspects of bacterial virulence, including facilitating adherence, colonization, chemotaxis, biofilm formation, the secretion of virulence factors, invasion, activation of the host immune defense mechanisms,[4] and antibiotic resistance.[5]

Recently, researchers have shown an increased interest in both intact flagellar structure[6] and motility[7] in bacterial pathogenicity, and multiple genes are involved in regulating and encoding them.[8] The *FleN* gene, known as the flagellar number regulator, has an effect on the number of flagella and chemotactic motility.[9] Additionally, the main global transcriptional regulator of flagellar genes[10] and biofilm genes, *FleQ*, is affected by *FleN*.[11,12] On the other hand, the *FlgE* gene (the flagellar hook gene), a structural gene responsible for encoding the hook portion of the flagella, affects

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flagellar motility, the synthesis of intact flagella,[13] and antibiotic resistance.[5]

The opportunistic bacterium *P. aeruginosa* is accountable for a diverse spectrum of serious severe illnesses, including wound infections, endocarditis, urinary tract infections, otitis media, burn-related infections, respiratory tract infections, nosocomial infections, bacteremia, and infections occurring in individuals with compromised immune systems.[14] Therefore, it regarded as one of the leading causes of mortality and morbidity in hospitalized patients.[15]

Materials and Methods

Specimens 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 Shahid Al-Sadr Hospitals, where 102 different specimens were collected. The specimens were collected from wounds, burn wound swabs, mid-stream urine, ear swabs, and sputum.

Isolation and identification of *Pseudomonas aeruginosa*

Conventional microbiological techniques, including cultivation on MacConkey agar for 24 h at 37°C, cetrimide agar for 24 h at 37°C and 24 h at 42°C, catalase and oxidase biochemical tests, and gram staining, were employed for the initial identification of bacterial isolates as *P. aeruginosa*. Subsequently, to confirm the identity of the bacterial isolate, Vitek 2-Compact was employed.

Molecular Assay

Primers designed and prepared

In the current study, the reference genes (*FleN* and *FlgE*) were downloaded from GenBank as FASTA files. The Geneious Prime tool was used for primers design, determining the proper annealing temperature and binding location for each pair of primers. Additionally, the primers were verified using a variety of online tools, primarily OligoAnalyzer from integrated DNA technology.

To create a stock solution containing 100 pmol/μL of primer, nuclease-free water was used to dissolve the primers. To create a working primer solution with a concentration of 10 pmol/μL, this stock was diluted by adding 10 μL of primer stock solution to 90 μL of nuclease-free water. The sequence of the primers was described in Table 1.

Extracting DNA

The EasyPure Genomic DNA Kit (Transgene, China) was used to extract the DNA from 33 *P. aeruginosa* isolates. Qubit 4.0 (ThermoFisher, USA), a high-selectivity assay, was then used to estimate the concentration of extracted DNA to determine whether the specimens were suitable for further downstream applications. The isolated DNA was then stored at −20°C until it was needed for subsequent use.

Molecular detection of FleN and FlgE genes

PCR was done using a thermocycler (ThermoFisher, USA), adding 12.5 μL of OneTaq® Two X Master Mix (NEB, England), 5 μL of the DNA specimens, 1 μL of each primer at a concentration of 10 pmol/μL, and 4.5 μL of free-nuclease water. The mixture was well mixed and vortexed to have homogeneous contents. The adjustment of the cycling program was: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 45 s for 50°C, 45 s for 72°C, and 7 min for the final extension at 72°C. Then, the electrophoresis gel tray wells were loaded with 10 μL of PCR product and DNA ladder and ran at 80 V for 80 min.

Statistical analysis

For data entry and analysis, the Statistical Package for the Social Sciences (SPSS/ version 21, Software, San Diego, CA) application was utilized. The mean, percentage, and standard deviation of the mean were employed to summarize all the data. In this cross-sectional study, the Chi-square test was utilized to determine the significance level of the categorical variables. $P < 0.05$ was regarded as statistically significant, while $P < 0.01$ was regarded as extremely significant.

Ethical approval

The ethics committee of the College of Science, University of Baghdad, approved this work (Ref. CSEC/0922/0072).

Table 1: Primers utilized in the current study

Primer name		Sequence (5′→3′)	Product size (bp)	Annealing temp. (C)	Reference
FleN	F	5′-TTCGACGAAGAACTCCAGGTGCC-3`	175 bp	58°C	Newly designed in this study
	R	5′-TGTAGGTGTCATCCCTTACGACGAGT-3′			
FlgE	F	5′-TGAACATCTGCGAGACGTCCGAG-3′	153 bp	59°C	
	R	5′-ACGTCACCGGCAACAACATCG-3′			

All participants agreed to provide the investigator with the specimens. The specimens were collected after the approval of the patients.

Results

Isolation and identification of *Pseudomonas aeruginosa*

From the total collected specimens, only 33 (32.35%) of them diagnosed as *P. aeruginosa*, as shown in Figure 1.

Pseudomonas aeruginosa produces round, mucoid, smooth, and pale colonies on MacConkey agar that smell like sweaty grapes. On Cetrimide agar, bacterial colonies appeared greenish-yellow. As shown in Figure 2.

Gram staining showed that the bacteria were Gram-negative bacilli as clarified in Figure 3.

Catalase and oxidase results have been positive, the result revealed in Figure 4. All 33 isolates have been confirmed to be *P. aeruginosa* by the Vitek 2-test.

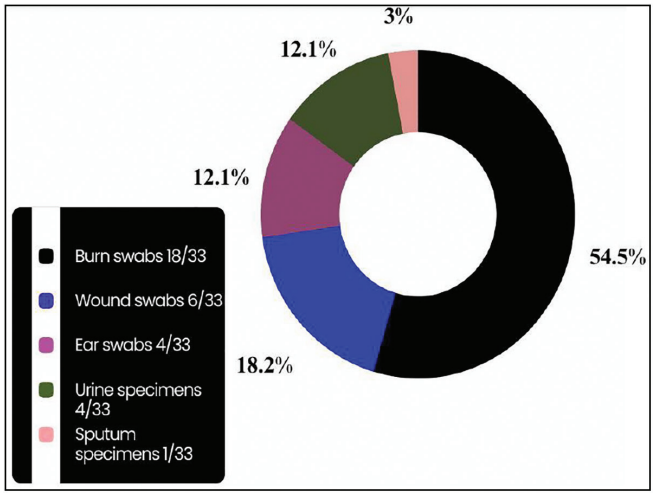

Figure 1: Isolation percentage of bacteria according to specimen source

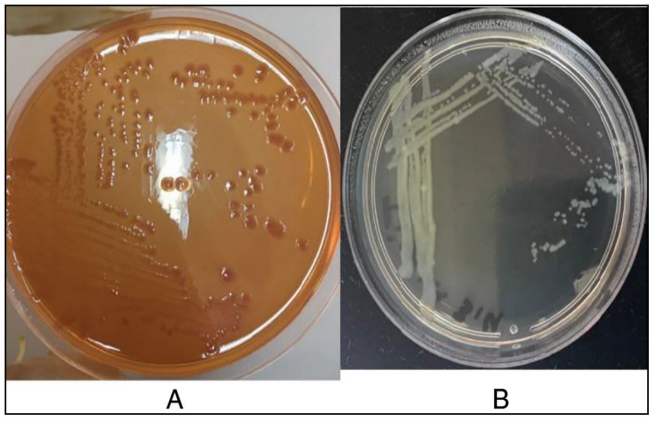

Figure 2: *P. aeruginosa* on (A) MacConkey agar, (B) Cetrimide agar for 24 h at 37°C

Molecular Assay

Molecular detection of *FleN* and *FlgE genes*

FlgE gene was found in twenty out of 33 (60.6%) isolates, while *FleN* gene was detected in 26 (78.8%) isolates, as shown in Figure 5 and Table 2.

Discussion

From the total collected specimens, only 33 were identified as *P. aeruginosa*. The development of a pale-yellow

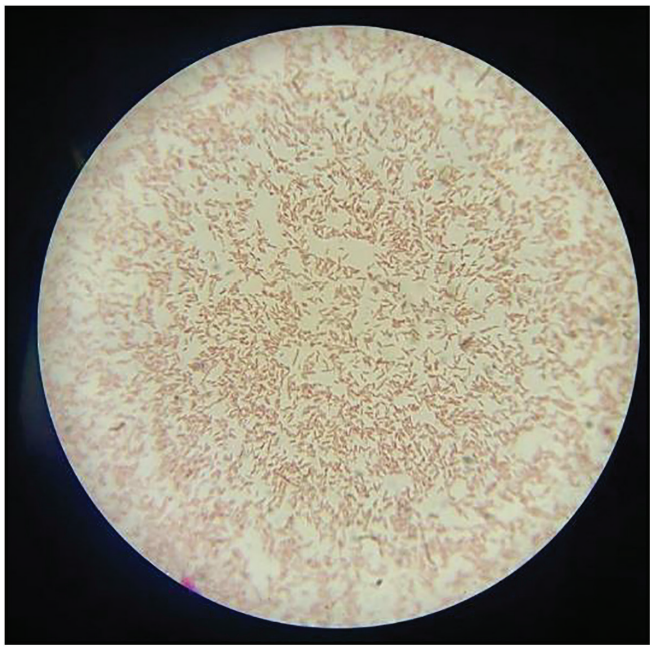

Figure 3: Microscopic field showing gram-negative rods

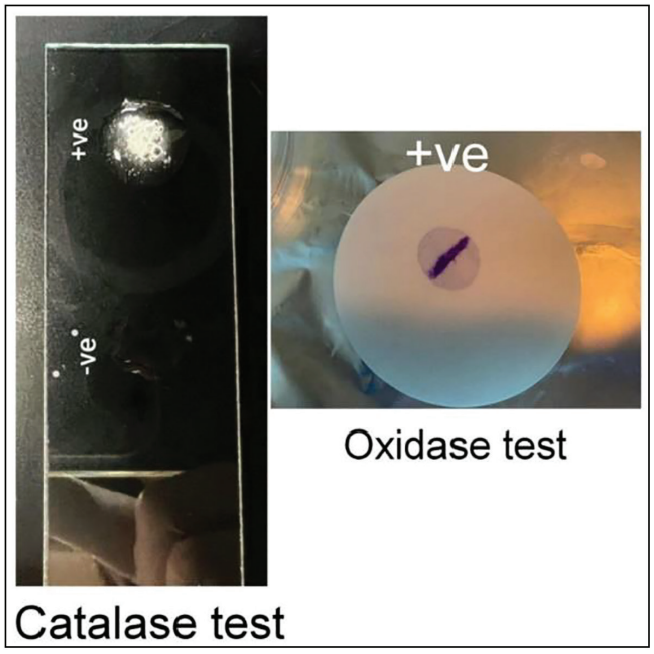

Figure 4: Biochemical tests

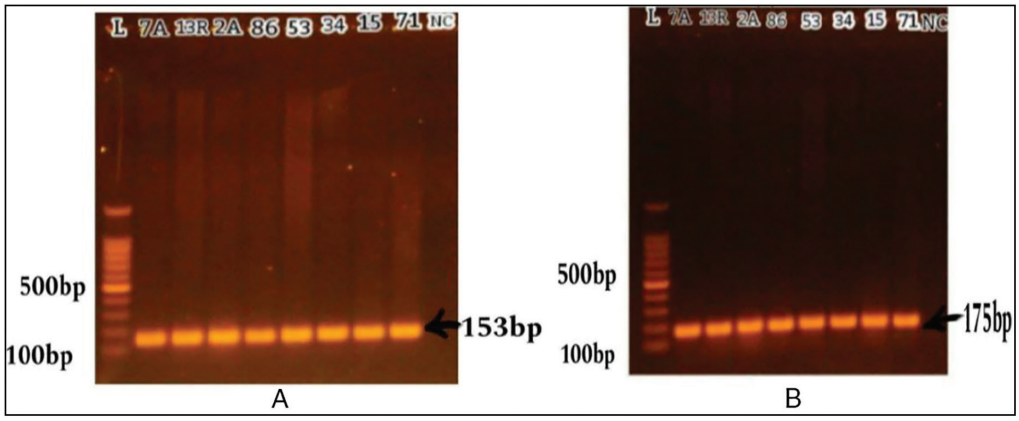

Figure 5: Results of the amplification of (A) *FlgE* and (B) *FleN* genes in *P. aeruginosa* isolates were fractionated on 2% agarose gel electrophoresis (80 V for 80 min) stained with red safe. L: 100 bp ladder marker. Numerated lanes resemble 153 bp for (A) and 175 bp for (B) PCR products

Table 2: Distribution of *FleN* and *FlgE* genes according to site of infection/ specimen

Site of infection/ specimen	Total isolates (%)	*FleN* gene (%)	*FlgE* gene (%)
Burn	18 (54.55)	16 (61.54%)	12 (60.00%)
Wound	6 (18.18)	4 (15.38%)	3 (1.00%)
Urine	4 (12.12)	3 (11.54%)	1 (5.00%)
Ear	4 (12.12)	2 (7.69%)	3 (15.00%)
Sputum	1 (3.03)	1 (3.85%)	1 (5.00%)
Total	33	26/33 (78.8%)	20/33 (60.6%)
Chi-square-χ^2 (*P* value)	---	29.001* (0.0001)	21.00* (0.0001)

* ($P \leq 0.01$) highly significant

colony with a sweaty grape odor on MacConkey agar due to lactose is a non-fermenter. Whereas they appeared greenish-yellow on the selective *Pseudomonas* Cetrimide medium. The selectivity of this medium is due to the presence of cetrimide, a quaternary ammonium compound with detergent-like properties that inhibits most bacterial species except *P. aeruginosa*, which can withstand toxic cetrimide material.[16]

Gram staining revealed that they were gram-negative bacilli. Also, all selected isolates showed the ability to grow at 42°C; this test is crucial for differentiating between fluorescent Pseudomonads species and non-pigmented *P. aeruginosa*.[17] The cultural characteristics and microscopic examination results agree with other study.[18]

All isolates were catalase positive, which means the presence of the catalase enzyme, which catalyzes the release of oxygen from hydrogen peroxide. This test is employed to distinguish between bacteria that produce the catalase enzyme and non-catalase-producing bacteria.[19] The study isolates also give positive results to the oxidase test based on the synthesis of the cytochrome oxidase enzyme, which catalyzes and transfers electrons between electron donors and a redox dye.[20] The oxidase test is used to distinguish Pseudomonadaceae from Enerobacteriaceae.[21]

According to the Vitek 2-test result, the study isolates had up to a 97% probability of being *P. aeruginosa,* which is an excellent diagnostic value.[22] It has been demonstrated in the past that the Vitek 2 diagnostic method is more rapid and labor-saving than conventional biochemical techniques.[23]

The results shown in the Figure 1 show that clinical specimens from burns had the highest percentage of isolation, followed by wound swabs, ear swabs, mid-stream urine, and sputum, respectively. Burn injuries are more vulnerable to opportunistic bacterial colonization by both endogenous and foreign bacteria because burns cause skin degeneration, which is the anatomical barrier and the body's first line of defense.[24] Sputum had the lowest isolation percentage of the total. The study's findings supported prior research.[25]

Although many studies investigated about the *FleN* and *FlgE* genes in *P. aeruginosa*, almost no single study has used conventional PCR to detect these genes in clinical isolates. The highest frequency of flagellar genes presence was shown in burn isolates, while the least frequency was in ear and urine isolates, followed by sputum isolates. Currently, there is a lack of research that investigates the presence rates of these genes in clinically isolated *P. aeruginosa.*

This pathogenic bacterium contains multiple flagellar genes, categorized as either regulatory or structural.[26] Among these genes is the *FleN* gene, which plays a critical role in regulating the number of flagella within *Pseudomonas* bacteria. Maintaining an appropriate number of flagella is an important task for motile bacteria, and the *FleN* gene significantly influences bacterial movement and, consequently, their pathogenicity.[27] Regarding the *FlgE* gene, it is a structural gene that encodes the hook part of the flagellum[28] and has a role in the synthesis and movement of the flagellum,[13] improving antibiotic resistance in the biofilm, and having an effect on bacteria adherence[5] thus contributing to the pathogenicity of *P. aeruginosa*.

Conclusion

Taking all of these research results into account, it becomes evident that the pathogenic bacterium *P. aeruginosa* is notably prevalent in burns and various clinical sites, as these isolates show a high percentage of the flagellar genes *FleN* and *FlgE* prevalence. These genes are pivotal in facilitating flagellar motility and the synthesis of a structurally intact flagellum, thereby significantly contributing to *P. aeruginosa* pathogenicity.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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