Iraqi Journal of Biotechnology, 2025, Vol. 24, Special Issue 2, 599-605

Source Tracking of *Pseudomonas aeruginosa* Isolated from Different Sources According to Flic Gene

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Received: February 20, 2025 / Accepted: May 20, 2025 / Published: November 16, 2025

Abstract: Pseudomonas aeruginosa is an aerobic, Gram-negative bacteria that is a leading cause of food contamination and nosocomial disease. For this research, 50 isolates were selected out of 73 total collected samples. Definitive identification of P. aeruginosa was based on characteristic morphological features of colonies, phenotypic and microscopic examinations on selective and differential media, in addition to standard biochemical tests. Based on these analyses, 25 clinical isolates were confirmed as P. aeruginosa, obtained from burns, urine, ear swabs, sputum, and wound infections. The remaining 25 isolates were collected from non-clinical sources, specifically white cheese and water samples.Genotypic confirmation was conducted using conventional PCR targeting the 16S rDNA gene with specific primers (pa-ss), resulting in 100% (50/50) positivity. Further molecular detection focused on the flagellin gene (fliC), a major virulence factor associated with motility and pathogenicity in P. aeruginosa. PCR results showed that 39 out of 50 isolates (76%) were positive for the fliC gene using specific primers.

Keywords: Pseudomonas aeruginosa, 16SrDNA, Flagillin, Flic.

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Introduction

Pseudomonads are a group of Gram-negative bacteria belonging to the family Pseudomonadaceae, which include several opportunistic species. They are motile via polar flagella and are known for producing both fluorescent and non-fluorescent pigments. (1). Pseudomonas spp. significantly contributes to milk spoilage and water pollution. During the storage of raw Various thermo-tolerant lipolytic and proteolytic enzymes are generated in milk, leading to a decline in the nutritional value and lifespan of processed milk (2). Consequently, due to the substantial emphasis on the species Pseudomonas and its pivotal role in food spoilage, nosocomial infections (NI) often arise in burn patients because of the disease's distinctive characteristics. Failure of the major defence mechanism against microbial invasion, a vascularised tissue that fosters an environment conducive to microbial multiplication. (3).The fliC gene encodes flagellin, the structural protein that makes up the filament of bacterial flagella. In Pseudomonas aeruginosa, this gene plays a crucial role in motility, allowing the bacterium to swim and spread across surfaces. Beyond motility, fliC is also involved in host-pathogen interactions, acting as a pathogen-associated molecular pattern (PAMP) that can trigger immune

responses in the host. Its presence is often associated with enhanced virulence, increased colonization ability, and persistence in both clinical and environmental settings. (4)

P. aeruginosa is now known as a significant threat among nosocomial infections, recognized as an opportunistic pathogen that targets immunocompromised individuals (5).It is the primary cause of surgical wounds, ear infections, and urinary tract infections (6). An efficient and precise approach for identifying Pseudomonas is crucial to avoid the future dissemination of infections by isolating patients. The cultivation of bacteria is a crucial technique still employed In diagnostic microbiology, this method is valuable for quantifying viable bacteria in a sample and for providing a pure sample for subsequent analysis (7). Consequently, it is imperative to establish genotype-based classification methods that can precisely categorise these microbes regardless of phenotypic alterations. DNA markers enable the swift determination of species. The Polymerase Chain Reaction (PCR) is an exceptionally sensitive, precise, and swift technique. among DNA markers that markedly improves The identification of P. aeruginosa, particularly through the use of species-specific primers such as 16S rRNA (8). The specific amplification of the Pseudomonas 16S rRNA gene through PCR has been employed to distinguish between Pseudomonas species in both clinical and environmental samples (9). This method is utilized for identifying P. aeruginosa at both the genus and species levels. (10). This organism is motile by a single polar flagellum An appendage resembling a whip is crucial for movement, encompassing both swimming and chemotaxis (11). The flagellum is recognized for its role as a virulence factor in the pathogenicity of various bacteria, such as P. aeruginosa (12). FliC flagellin plays a vital role in bacterial chemotaxis during infection, aiding the bacterium's attachment to host epithelial cells and initiating a strong The inflammatory response is mediated by the nuclear factor kappa-light-chain enhancer of activated B cells (NF-kB) via TLR5 signalling. Furthermore, it triggers a response mediated by caspase-1 through the Nod-like receptor (NLR) and the Interleukin-1β-converting enzyme (ICE) protease-activating factor (Ipaf) (13).

Materials and Methods

Sample collection:

Samples were collected from the commercial market in Baghdad city, and clinical samples were sourced from patients with infected wounds, which included ear infections, burns, and other types of wounds. Furthermore, sputum and urine samples were obtained from patients with a range of illnesses in hospitals throughout Baghdad, in addition to food samples.

Identification of the phenotype of P. aeruginosa

As noted by (14), the swabs underwent enrichment in brain-heart infusion broth before being plated on to MacConkey agar, blood agar, nutrient agar. A single colony was designated and inoculated on The selective medium comprises cetrimide agar and Pseudomonas agar. Following this, the phenotypic characteristics of P. aeruginosa were identified following Gramme staining, which included the production of pigments after incubation at 37ºC. They consist of several "cassettes" that each include genes for antibiotic resistance. It significantly contributes to bacterial multi-antibiotic resistance development. As a result, Bacteria possess the ability to adapt and endure

in environments where antibiotics are present. (15).

DNA Extraction

Preparation of genomic DNA

The purification of bacterial genomic DNA was performed using a Wizard Genomic DNA Purification Kit from Promega. The bacterial culture was transferred into 1.5 ml microcentrifuge tubes and centrifuged for 2 minutes at 13000-16000x g, following which the supernatant was discarded. The supernatant was stored at -20◦C to serve as a template DNA stock.

Application of PCR

Fifty Samples of P. aeruginosa were selected for examination utilizing PCR methodologies.The PCR reaction mixture was meticulously prepared in a sterile 0.5ml Eppendorf tube, consisting of: a PCR mixture for this gene made up of 12.5µl of GoTaq®Green Master Mix, forward and reverse primers (1.25µl each), template DNA at 5µl, and 5µl of Deionized D.W. was additional to PCR mixture to get last volume of 25µl. PCR mixture without template DNA was used as a negative control, using a thermal Cycler (Gene Amp, PCR system 9700, Applied Biosystem). Primer pair *PA-SS*-F (5'GGGGGATCTTCGGACCTCA 3') and *PA-SS*-R (5' TCCTTAGAGTGCCCACCCG 3') was accustomed amplify *P. aeruginosa* specific DNA. Which designed which amplifies 956 bp. The amplification program consisted of as follows: One pre-cycle at 95°C for 2 minutes, conducted for 30 cycles (92°C's for one minute, 55 - 59°C's for 1 minute based on the specific primer applied, and 72 degrees Celsius for 1 minute), concluding with a final extension phase at 72°C for ten minutes.. As demonstrated in tables 1 and 2. Identification of pathogenicity genes flagellin gene Flic A collection of conserved oligonucleotide primers, Flic CW45 and CW46, was utilised to examine flagellin subtypes in the clinical strains.

Table (1): primers and sequence of flic gene

Primer Name	Sequence (5' → 3')	Amplicon Size	Type
fliC-F (CW45)	GGCAGCTGGTTNGCCTG	1,020 bp	Type a
fliC-R (CW46)	GGCCTGCAGATCNCCAA	1,250 bp	Type b

Table (2): PCR Amplification Program

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	2 minutes	1 cycle
Denaturation	95°C	40 seconds	30 cycles
Annealing	55°C	1 minute	
Extension	72°C	2 minutes	
Final Extension	72°C	10 minutes	1 cycle

Results and Discussion

P. aeruginosa is frequently detected in contaminated food and environmental settings, recognized as an opportunistic pathogen.This study involved the selection of 50 clinical P. aeruginosa isolates obtained from both the commercial market and hospitalised patients.

73 isolates were grown on agar from MacConkey, nutrient agar, Pseudomonas agar, and Citrimide agar.After a 24-hour incubation period at 37°C, a total of 50 P. aeruginosa were confirmed via biochemical testing, while the other 23 isolates were determined not to belong to this species. Out of a total of 50isolates, 46 yielded positive results on cetrimide agar containing nalidixic acid. This antibiotic is valuable for the The process involves the the process of isolating and

identifying P. aeruginosa, differentiating it from other species within the Pseudomonas genus species, particularly due to its resistance characteristics. this antibiotic, while others demonstrate sensitivity to it (16).

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Identification of strains by the isolation of genetic DNA from bacterial isolates

revealed that the extracted DNA, as shown in Figure (1), demonstrates that this protocol is an effective method for DNA extraction from P. aeruginosa.

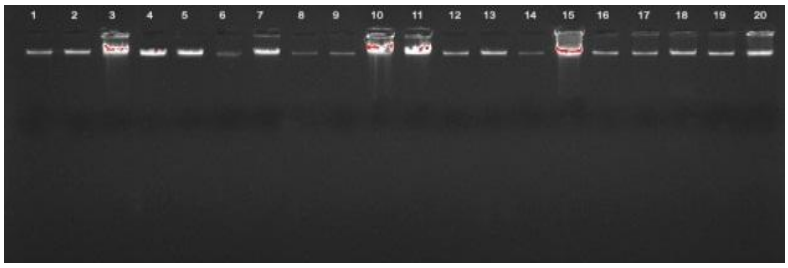

Figure (1): Agarose gel electrophoresis of bacterial genomic DNA using 0.7% agarose, TBE (1x) and visualized by ethidium bromide stain.

Analysis of bacterial isolates using PCR

In the study, both primer pairs were employed; the pair (Pa-SS-F and Pa-SS-R) was specifically crafted for P. aeruginosa. The P.C.R. assays utilising this primer pair yielded DNA products of the anticipated size, as shown in Figure (2). The findings indicated that all 50 isolates yielded positive results.

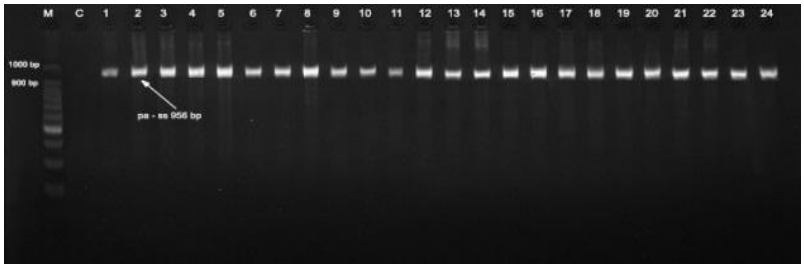

Figure (2): A 956 bp PCR amplification of 16SrDNA specific for P. aeruginosa was detected in each sample through the application of 1.5% (1 hours /70 vol) agarose gel electrophoresis. M 1000kb DNA ladder, c: Negative control and samples 1-24 consisted of various isolates of P. aeruginosa.

The Flic gene comprises two variants: type A, which possesses a molecular weight of 1020 base pairs, along with type B, showing 1250 base pairs (17). The The findings of our study indicated that all isolates were positive for the 16S rRNA gene. Thirteen isolates exhibited type A, while nine isolates displayed type B of the gene; however, there were three that did not conform to these classifications. isolates that did not have a *flic* gene when the food in 17 isolates gave positive result and 8 gave negative . These were result of isolate is agreement with (18). food and clinical isolates, we performed a comparative analysis of fliC gene types:A significant correlation ($p < 0.05$) was observed between the distribution of fliC gene types in foodisolates and clinical isolates, indicating a possible common source or transmission route.Particularly, fliC type A was dominant in both food and clinical samples, supporting potential epidemiological linkage. The absence of fliC in some food isolates, while present

in clinical isolates, may suggest gene loss, variation in gene expression, or sampling differences.This genetic similarity suggests a strong potential for foodborne transmission of the pathogen responsible for the clinical cases As showed in table (3).

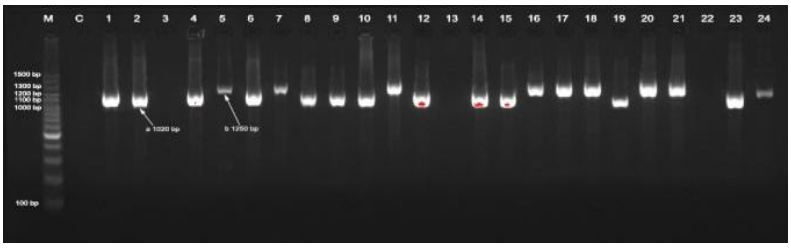

Figure (3): Agarose gel electrophoresis of PCR product amplified from the flic gene. The DNA fragments of type a contain 1020 bp and type b estimate 1250 bp, both amplified from the flic gene using a ladder. Lane M represents the 100-1500 bp marker, while lane C serves as the negative control. The figure indicates that all samples yielded positive results (lanes 1 to 24), with the exception of three isolates that produced negative results.

In addition to extracellular factors, the initial accessory mediator (flagella) is crucial in the onset of infection. Two distinct types of flagellin proteins have been identified in P. aeruginosa, designated as type 'a' and type 'b'. These types can be distinguished based on their molecular size and their reactions with type-specific monoclonal and polyclonal antibodies (19). Type 'a' and 'b' flagellin of P. aeruginosa do not exhibit phase variation; a solitary strain produces a single type of flagellin, and no switching between types 'a' and 'b' has been experienced. Oligonucleotide primers specific for N-terminal (CW46) and C-terminal (CW45) preserved regions of flagellin gene were used for PCR amplification of the flagellin gene of *P. aeruginosa* PAO1. In a physical genome analyze of the virulence-associated *fliC* locus in *P. aeruginosa* strains, charting and sequencing revealed collections of heterologous a-type (1164 bp; 1185 bp) and highly preserved 'b'-type (1467 bp) flagellin genes. *FliC* occurrence was found to be 90% (37.77% '*b*'-type flagellin, 62.23% '*a*'-type) (20).

Table (3): Genetic Comparison of fliC Gene Between Food and Clinical Isolates

fliC Gene Type	Food Isolates (n)	Clinical Isolates (n)	Total (n)	Observation
Type A	12	13	25	Most common in both groups
Type B	5	9	14	Present in both groups, less frequent
Absent	8	3	11	Some isolates lacked the fliC gene
Total	25	25	50	

Conclusion

The results of this study demonstrate a notable presence of the fliC gene in both food and clinical isolates, with fliC type A being the most prevalent. This suggests a potential genetic link between foodborne sources and clinical infections. The detection of the same fliC types in both groups highlights the role of contaminated food as a possible vector for transmission. Furthermore, the absence of the fliC gene in a subset of isolates may indicate gene variability, deletion events, or

differences in bacterial strains, warranting further genetic investigation.

Whole Genome Sequencing (WGS): To better understand the genetic relatedness and possible clonal relationships between food and clinical isolates.

Functional Analysis of fliC: Investigate the role of fliC gene expression in virulence, motility, and host interaction.

Surveillance Studies: Broader sampling from different food sources and clinical settings to monitor the distribution of fliC variants over time.

Resistance Correlation: Study possible associations between fliC gene types and antimicrobial resistance profiles.Risk Assessment Models: Incorporate genetic data into quantitative microbial risk assessment models to evaluate public health risk from foodborne pathogens.

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