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Research Article

Molecular detection of *toxA* and *lasB* virulence genes of *Pseudomonas aeruginosa* strains isolated from Diabetic Foot Ulcers in Erbil, Iraq

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Abstract
Diabetic foot ulcers (DFUs) are a major cause of lower limb medical amputations and result in high healthcare expenses. In DFUs, *Pseudomonas aeruginosa* is significantly prevalent. The current study aims to determine the relationship between *toxA* and *lasB* virulence genes of *Pseudomonas aeruginosa* and antibiotic resistance. Between September 2024 and February 2025, a total of 150 swabs were collected in this cross-sectional study from DFU patients of both sexes and varying ages who attended the American Medical Complex for treatment. The primary identification was performed using cultural characteristics and biochemical tests, and the definitive identification was achieved using the Vitek 2 compact system. Antimicrobial susceptibility tests were done by the Disk diffusion method. Of 150 samples, 53 (35.33%) were identified as *P. aeruginosa*. Among these, the prevalence of the *toxA* and *lasB* genes was 90.56% (48/53) and 88.68% (47/53), respectively. Ceftazidime was the most effective antibiotic, followed by netilmicin, meropenem, and imipenem. Carbenicillin was the least effective (100% resistance), followed by levofloxacin, piperacillin, ciprofloxacin, and cefepime.P. aeruginosa was prevalent in DFUs, accounting for 35.33% of the total sample size. Ceftazidime is the most effective antibiotic for treating *P. aeruginosa* infected DFUs, followed by netilmicin, meropenem, and imipenem. Carbenicillin is less effective. The toxA and lasB virulence genes was highly prevalent in *P. aeruginosa* isolates, leading to increased antibiotic resistance. This study provides insights into the virulence and resistance of *P. aeruginosa* in diabetic foot ulcers, emphasizing the need for molecular monitoring and customized antibiotic therapies to improve clinical management.

Keywords: Antibiotic susceptibility, Diabetic foot infections, Diabetic foot ulcer, *Pseudomonas aeruginosa*, Virulence genes

INTRODUCTION

Diabetes Mellitus (DM) stands as the most prevalent endocrine disorder globally. Individuals with diabetes have an increased susceptibility to pneumonia, urinary tract infections, and skin and soft tissue infections (Al-Khashmani *et al*., 2021).

Diabetic foot ulcers (DFUs) represent significant complications of DM, resulting in osteomyelitis, gangrene, and potential limb amputation. The observed outcomes elevate mortality risk and contribute to bacterial resistance among surviving patients (Abbas *et al*., 2024). It primarily comprises various pathological conditions characteristic of the diabetic foot. The pathologies include foot infections, foot ulceration, diabetic peripheral neuropathy, and diabetic peripheral vasculopathy, also known as peripheral artery disease (PAD). The progression of events leading to diabetic foot ulceration in patients with DM begins with lower limb peripheral neuropathy(PN) (Tuttolomondo *et al*., 2015). This directly led to a reduction in the capacity for pain sensation, which correlated with the delayed identification of small or minor wounds and injuries. This situation may be associated with inadequate blood circulation in the lower limb due to PAD, resulting in prolonged healing times for minor wounds and an increased risk of wound infection (Bakker *et al*., 2012).

Diabetic foot infections pose a significant risk for hospitalization and amputation (Al-Chalabi *et al*., 2024). The majority of diabetic foot infections are preceded by foot ulcers. Patients with recurrent, long-standing, or bone-probing lesions, as well as those with a recent history of non-foot infection, are at a higher risk (McDermott *et al*., 2023). Pathogenic microbes

frequently isolated from these wound areas include *Staphylococcus, Pseudomonas, Klebsiella, and Escherichia coli*. *Pseudomonas aeruginosa* is consistently linked to DFUs (Srivastava and Sivashanmugam, 2021).

P. aeruginosa is a prevalent opportunistic pathogen in humans, associated with numerous acute and chronic diseases (Hussein, 2021). It is a rod-shaped, aerobic, Gram-negative bacterium (Al-Zwaid and Al-Dahmoshimoshi, 2022). *P. aeruginosa* is motile bacterium by a polar flagellum (Kamal *et al*., 2018). This bacterium is widespread and can survive in both living and non-living environments. It is capable of withstanding temperatures between 4°C and 42°C (AL-Samaraey and Jafar, 2021). *P. aeruginosa* is among the most adapTable and pathogenic opportunistic bacterial infections identified to date. It is a primary etiological agent of bacteremia and sepsis in individuals undergoing cancer treatment, the predominant cause of nosocomial pneumonia, a common pathogen in diabetic ulcers, burn wounds, surgical incisions, and corneal ulcers, as well as a lethal contributor to chronic infections in cystic fibrosis patients (Karim *et al*., 2023; Wood *et al*., 2023). *P. aeruginosa* generally exhibits reduced susceptibility to various antibiotics compared to other Gram-negative bacilli. Multidrug-resistant *P. aeruginosa* poses significant clinical challenges and is a matter of considerable concern (Abd Al Zwaid and Al-Dahmoshi, 2022). *Pseudomonas aeruginosa* possesses numerous virulence factors, including exotoxin A, exoenzyme S, and elastases, all of which are intricately regulated by cell-to-cell signalling systems (Liao *et al*., 2022).

Exotoxin A(ETA) represents the primary virulence factor of *P. aeruginosa* and is encoded chromosomally (Morgan *et al*., 2023). ETA is heat-labile, consisting of 613 amino acids, with a molecular weight of 66 kilodaltons, and is encoded by the *toxA* gene (Abd Al-doori *et al*., 2020). This toxin facilitates the transfer of ADP ribose from nicotinamide adenine dinucleotide (NAD+) to diphthamide, thereby suppressing protein synthesis and ultimately causing the death of eukaryotic cells through the inhibition of this process (Jawad and Rasheed, 2022).

Pseudomonas elastase B, referred to as LasB or pseudolysin, is a Zn2+-dependent metalloprotease exhibiting neutral characteristics, which is encoded by the *lasB* gene. LasB is characterized by its elastolytic and exoprotease activity. LasB has demonstrated the capacity to hydrolyze various host proteins, resulting in tissue damage, immune response disruption, and inflammation promotion (Everett and Davies, 2021). The current study aimed to determine the relationship between *toxA* and *lasB* virulence genes of *Pseudomonas aeruginosa* and antibiotic resistance isolated from DUFs in Erbil, Iraq.

MATERIALS AND METHODS

Sample collection

The samples were collected from patients with DFU by taking wound swabs from ulcers in the infected foot. A total of 150 DFU patients of both sexes and varying ages (25-85) year and varying resident (urban and rural) were included in the present study.

Culture media and preliminary identification

This study utilized various media and implemented several tests, including blood agar, MacConkey agar, Cetrimide agar, Muller-Hinton agar, Simmon's citrate agar, Kligler iron agar, indole production test, peptone water, methyl red test, Voges-Proskauer test, urease, catalase, and oxidase tests, as well as the Gram stain. The media were prepared based on the manufacturers' specifications (Ikken *et al*., 2021; MacFaddin, 2000).

The swabs were streaked on blood agar and MacConkey agar and incubated at 37°C for 18–24 hours in an incubator. Positive cultures were sub-cultured on cetrimide agar (which is selective for *P. aeruginosa)*, and were subjected to Gram staining and biochemical tests for identification (Gilardi, 2020).

Definitive identification

Definitive identification of *P. aeruginosa* was done using (GN-ID) with the VITEK-2 compact system, manufactured by bioMerieux. The manufacturer's specifications were followed during the test process.

Antibiotic susceptibility test

Using commercially prepared antibiotic disks on Mueller Hinton agar plates, antimicrobial susceptibility tests were performed on isolated and identified colonies of *P. aeruginosa* using the disk diffusion method in compliance with the Central Laboratory Standards Institute (CLSI) guidelines 2024. The carbenicillin (100µg), piperacillin (100µg), ciprofloxacin (10µg), levofloxacin (5µg), norfloxacin (10µg), ceftazidime (30µg), cefepime (30µg), netilmicin (30µg), gentamicin (10µg), imipenem (10µg), and meropenem (10µg) were the antibiotics employed in present investigation. Different concentrations of antibiotics were standardized based on clinical relevance, pharmacokinetics, pharmacodynamics, and resistance breakpoints

DNA extraction

Genomic DNA extraction from *P. aeruginosa* isolates was done using the Geneaid genomic DNA purification kit (UK) and was produced it as directed by the company.

Detection of *toxA* and *lasB* genes

Primers designed to facilitate molecular screening for virulence genes in *P. aeruginosa* isolates being investi-

Table 1. Primers used in the present study

No.	Gene	Primer sequence (5'- 3')	Size
1	*tox*A	F GACAACGCCCTCAGCATCACCA R CGCTGGCCCATTCGCTCCAGCG	397bp
2	*las*B	F GGAATGAACGAAGCGTTCTC R GGTCCAGTAGTAGCGGTTGG	300bp

Table 2. Polymerase chain reaction (PCR) conditions to detect the *toxA* gene

Steps	Temp. °C	Time	Cycle
Initial denaturation	94	4 min	1
Denaturation	94	30 sec	35
Annealing	65.8	45 sec	35
Extension	72	60 sec	35
Final extension	72	5 min	1

Table 3. Polymerase chain reaction (PCR) conditions to detect the *lasB* gene

Steps	Temp. °C	Time	Cycle
Initial denaturation	95	3 min	1
Denaturation	94	30 sec	30
Annealing	50	60 sec	30
Extension	72	60 sec	30
Final extension	72	5 min	1

gated were used in a polymerase chain reaction (PCR) sequence. The sequences of the primers are presented in Table 1.

Formulated a 10 µl combination of GO Taq Green Master Mix as per the manufacturer's instructions, including 1 µl of F-Primer, 1 µl of R-Primer, 3 µl of DNA sample, and 5 µl of nuclease-free water. The ion-free nuclease water manufactured by Bioneer Korea, was then combined with the contents. PCR tubes were carefully prepared using a microcentrifuge spin and then inserted into a PCR apparatus, following the designated protocol for each primer according to their binding temperatures, as detailed in Table 2 and 3.

RESULTS

Preliminary identification

The analysis involved 150 specimens collected from patients with DFUs, encompassing both superficial and deep-seated infections across all patients. Out of these 150, 53(35.33%) isolates exhibited growth on cetrimide agar and were identified as *P. aeruginosa* based on several criteria: complete hemolysis on blood agar, non-lactose fermentation on MacConkey agar, Gram-negative, indole-negative, methyl red-negative, Voges-Proskauer-negative, citrate-positive, catalase-positive, Kligler iron alkaline/alkaline, H_2S-negative, urease-negative, oxidase-positive, growth at 42°C positive, and growth on cetrimide agar positive.

Males showed the highest frequency of DFUs with a recorded percentage of 70% as shown in Table 4. Regarding the distribution of DFUs by age groups, the highest occurrence of DFUs was observed in the 46-65 year age group, with a recorded percentage of 67.65%, as shown in Fig. 1.

The study showed that people residing in the urban area of Erbil, Iraq, had the highest prevalence of DFUs, with a recorded percentage of 54%, as shown in Table 5.

Definitive diagnosis: The 53 (35.33%) isolates were definitively identified as Pseudomonas aeruginosa using the VITEK-2 compact system (Fig. 2).

Antibiotic susceptibility test

The present results showed that ceftazidime was the most effective antibiotic against P. aeruginosa strains isolated from DFUs, with a sensitivity rate of 44 (83.01%), followed by netilmicin (32, 60.4%), meropenem (23, 43.4%), and imipenem (22, 41.5%). In the other hand carbenicillin was the less effective antibiotic because all *P. aeruginosa* isolates 53(100%) were resistant to it, followed by levofloxacin 42(79.24%), piperacillin 41(77.36%), ciprofloxacin 39(73.58%) and cefepime 37(69.81%) as illustrated in Fig. 3.

Polymerase chain reaction (PCR) results

As illustrated in Table 6. The study found the *toxA* gene present in 48(90.56%) isolates from the total isolates (53) of *P. aeruginosa,* see (Fig. 4), and *lasB* gene was present in 47(88.68%) isolates from the total isolates (53) of *P. aeruginosa* see (Fig. 5).

DISCUSSION

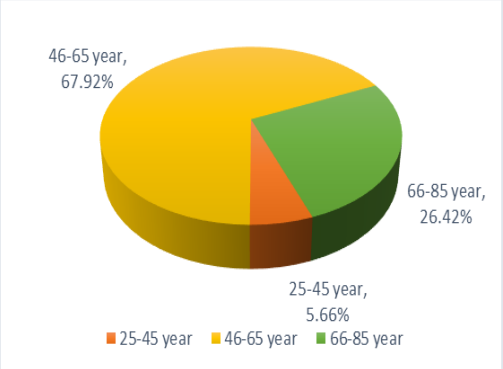

Fig. 1. *Distribution of diabetic foot ulcers (DFUs) in age groups*

Pseudomonas aeruginosa causes serious infections that can impact almost every organ in the human body, including lung infections, pneumonia, soft tissue infections related to burns and open wounds, urinary tract infections, keratitis, and DFUs. In the present study, the prevalence of *P. aeruginosa* in DFUs was 35.33% (Fig. 2). The nearest prevalence to our study, as far as we know, was observed in Al-Najaf City, Iraq (34.4%) (Alkhudhairy and Al-Shammari, 2020), and Abadan City, Iran (30.4%) (Rezazadeh *et al*., 2023). A lower prevalence rate was recorded in Hila City, Iraq (24%) (Abbas *et al*., 2024). The current study indicates a higher prevalence of DFUs in male patients compared to female patients, with rates of 70% and 30%, respectively (Table 4). Nearly similar percentages were reported in studies conducted in countries adjacent to Iraq, such as Kuwait (66.9% male, 33.1% female) (Alhubail *et al*., 2020) and Syria (63% male, 37% female) (Al Abbas *et al*., 2022). Men exhibit a greater likelihood than women of developing DFU, attributed to a higher prevalence of PN, PAD, and cardiovascular disease in diabetic males; also, women demonstrate a greater propensity for adhering to recommended self-care and foot care practices (McDermott *et al*., 2023). This study revealed a significant prevalence of *P. aeruginosa* among individuals aged 46-65 years, with a rate of 67.92% (figure 1). A study conducted in Al-Najaf, Iraq, indicated that the highest prevalence of DFUs was observed in the age group of 50-59 years, accounting for 39.2% (Alkhudhairy and Al-Shammari, 2020). In the current study, the prevalence of DFUs was higher in diabetic patients living in the urban area, with a recorded percentage (54% urban and 46% rural) (Table 5). A study has indicated that people living in rural areas or underprivileged communities may have a higher risk of developing DFUs (Schmidt *et al*., 2024). The variation between this study and the present study regarding the residence is due to the type of study and the targeted population.

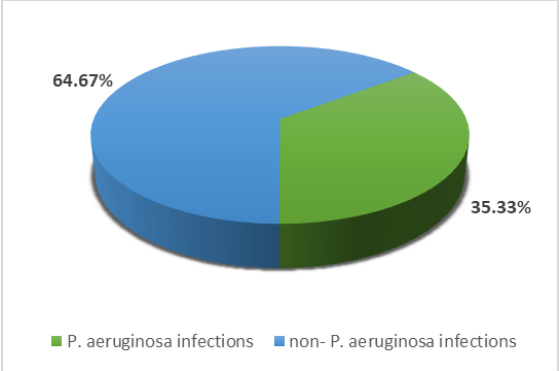

Fig. 2. *Frequency of Pseudomonas aeruginosa among Diabetic foot ulcer (DFUs) patients*

The current study demonstrated that *P. aeruginosa* exhibited sensitivity to ceftazidime (83%), netilmicin (60%), imipenem (43%), and meropenem (42%) (Fig. 3). A previous study conducted in Coimbatore, India, demonstrated a sensitivity to imipenem of 33.3% (Sivanmaliappan and Sevanan, 2011), In contrast, a study carried out in Sulaimani City, Iraq, revealed sensitivity patterns of 100% for imipenem, 90.2% for meropenem, and 52.9% for ceftazidime (Qadir *et al*., 2020). A prior study in Syria demonstrated a sensitivity of 100% to imipenem (Al Abbas *et al*., 2022). The variability in results from multiple studies suggests that antibiotic susceptibility patterns for treating *P. aeruginosa* isolated from DFU patients are inconsistent. Consequently, ongoing evaluations of microbial traits and antibiotic susceptibility are crucial for selecting the most appropriate antibiotics.

The pathogenicity of *P. aeruginosa* is enhanced by the ability to secrete various extracellular enzymes, including exotoxins, proteases, elastases, haemolysin, and nucleases (Morin *et al*., 2021). Exotoxin A is the primary virulence factor of *P. aeruginosa.* In the current study the results of the molecular amplification of these products showed that most isolates (48, 90.56%) had the *toxA* gene (Table 6). The same result is documented in Shiraz, Iran (90.4%) (Yousefi-Avarvand *et al*., 2015). In research in Poland, the prevalence of *toxA* in clinical isolates of *P. aeruginosa* was 88.7%, which is comparable with our study (Wolska and Szweda, 2009). A high percentage of prevalence of the *toxA* gene in *P. aeruginosa* isolated from DFUs suggests a strong relationship between *toxA* and the development and progression of DFUs, as *toxA* inhibits protein synthesis in the

Table 4. Distribution of diabetic foot ulcers (DFUs) according to sex

Sex	No.	%
Male	105	70
Female	45	30
Total	150	100

Table 5. Distribution of diabetic foot ulcers (DFUs) according to residents

Residents	Diabetic foot ulcer patients (DFU)	
	No.	%
Urban	81	54
Rural	69	46
Total	150	100

Table 6. Frequency of *Pseudomonas aeruginosa toxA* and *lasB* virulence genes in the distribution of diabetic foot ulcers (DFUs) samples

P. aeruginosa genes	Positive	Negative	Total
toxA	48	5	53
lasB	47	6	53

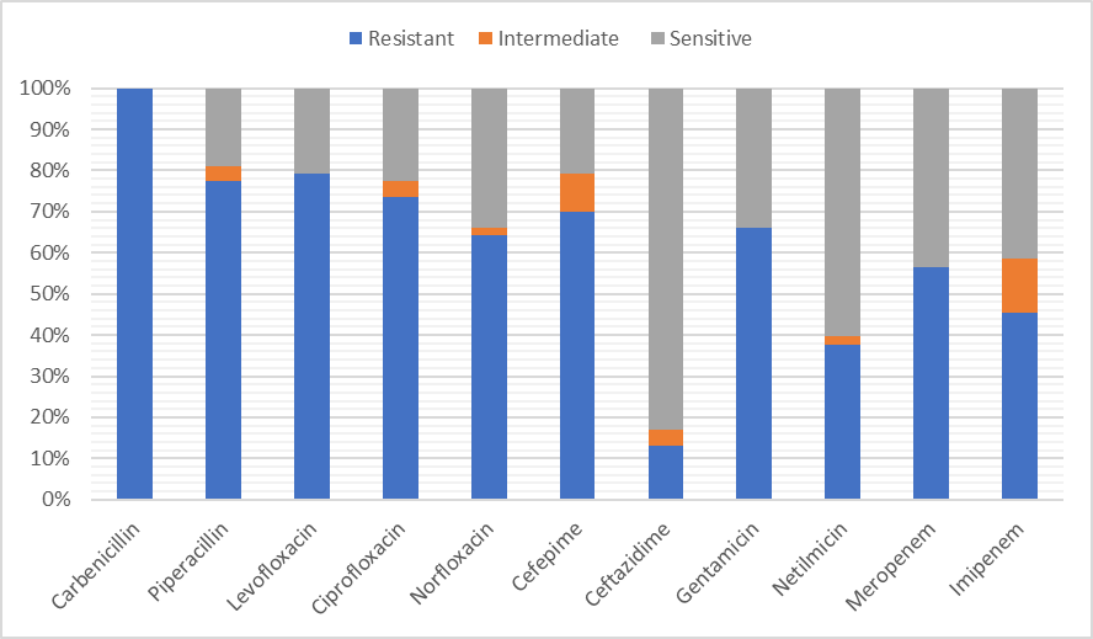

Fig. 3. *Susceptibility results of isolated Pseudomonas aeruginosa from Diabetic foot ulcers (DFUs)*

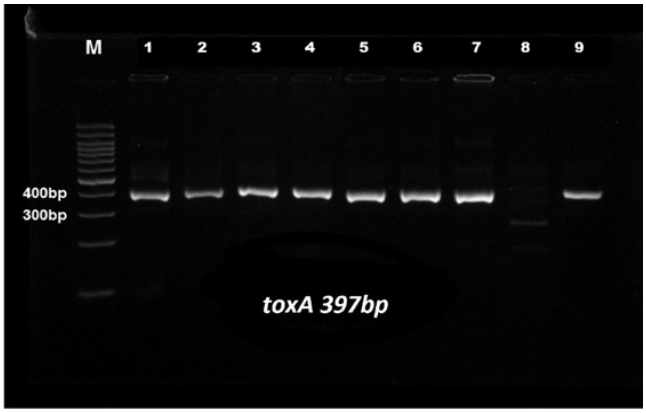

Fig. 4. *Agarose gel Electrophoresis was conducted on the Polymerase chain reaction (PCR) of extracted DNA from Pseudomonas aeruginosa isolates, utilizing specialized primers targeting the toxA gene with a concentration of 2% and a voltage difference of 60 volts, maintained for a duration of 2 hours; M track volumetric guide (100pb DNA ladder)*

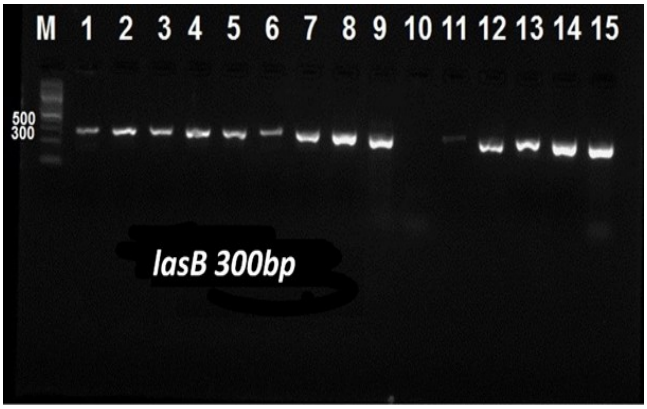

Fig. 5. *Agarose gel Electrophoresis was conducted on the Polymerase chain reaction (PCR) of extracted DNA from Pseudomonas aeruginosa isolates, utilizing specialized primers targeting the lasB gene with a concentration of 2% and a voltage difference of 60 volts, maintained for a duration of 2 hours; M track volumetric guide (100pb DNA ladder)*

host, leading to cellular destruction.

In the current study the results of the molecular amplification showed that most isolates (88.68%) carried the *lasB* gene (Table 6). A study conducted in Ardabil, Iran, showed that the prevalence of the *lasB* gene was 86.9% in clinical isolates (Bazghandi *et al*., 2021). In Iraq, a study revealed that the prevalence of the lasB gene was 94.6% in P. aeruginosa isolated from clinical samples (Al-Shimmary, 2020). Another study conducted in Baghdad, Iraq, revealed a 100% prevalence of the lasB gene. The elevated expression of elastase encoded by the *lasB* gene suggests its significance in the pathogenesis of this bacterium, particularly in the degradation of collagen and elastin. lasB plays a crucial role in the destruction of junctions between epithelial cells, altering their mobility and inhibiting cell injury. This makes *lasB* a very important virulence factor in the development and progression of DFUs.

It is essential to observe the fluctuations in antibiotic resistance and virulence genotypes of *P. aeruginosa*, which could offer sophisticated tools for more effective management of the infection. The present study results showed that *P. aeruginosa* strains with *toxA* and *lasB* were more resistant than strains without these genes. We had isolated one strain of *P. aeruginosa* from DFU that lacked *toxA* and *lasB*, this strain was sensitive to all antibiotics used except piperacillin and carbenicillin. In Mazandaran, Iran, a study showed similar results regarding the relationship between (*toxA* and *lasB*) virulence genes of *P. aeruginosa* and the development of

antibiotics resistant (Elahi *et al*., 2024). A study carried out in Shanghai, China, has shown that P. aeruginosa strains lacking the *toxA* gene were less resistant to antibiotics (Wang *et al*., 2025). To effectively treat P. aeruginosa infections and formulate effective management strategies, further studies are necessary to fully understand the relationship between antibiotic resistance and virulence variables.

Conclusion

Pseudomonas aeruginosa was prevalent in DFUs, with a recorded percentage of 35.33% of the total sample size. Ceftazidime was the most effective antibiotic for treating P. aeruginosa-infected DFUs, followed by netilmicin, meropenem, and imipenem. On the other hand, carbenicillin was the less effective antibiotic. The prevalence of *the toxA virulence gene was remarkably high in isolates of P. aeruginosa from DFUs, and strains with the toxA gene were more resistant to antibiotics than those without it*. The prevalence of the *lasB* virulence gene was significantly elevated in *P. aeruginosa* isolates from DFUs, with *lasB*-positive strains exhibiting greater antibiotic resistance compared to *lasB*-negative strains.

Conflict of interest

The authors declare that they have no conflict of interest.

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