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Molecular Detection of Beta-Lactamase Genes (KPC and CTX-M) of *Pseudomonas aeruginosa* in Diabetic Foot Ulcers Patients

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ABSTRACT

Background: *Diabetic foot ulcers (DFUs) are an inflammatory disease caused by a bacterial infection such as Pseudomonas aeruginosa (P. aeruginosa), provided with genetic virulence factors. This study aims to detect KPC and CTX-M genes of P. aeruginosa isolated from chronic DFU patients.* ***Methods:*** *One hundred twenty DFU patients were recruited from Al-Sader Medical City and Al-Hakim General Hospital in Najaf province from September to January 2021 to collect ear swaps. The Primary identification depends on Gram stain and biochemical tests. We also used the Vitek 2 system to make another identification and examine resistance to antibiotics. A polymerase chain reaction (PCR) was used to amplify the required genes. Furthermore, gel electrophoresis was performed to show the amplified genes.* ***Results:*** *The results have revealed that 100 samples (83.3%) give a positive outcome, while 20 samples (16.6%)are negative for culture bacteria. Only 17(17 %) of the 100 clinical specimens showed positive tests for P. aeruginosa. However, the gene, CTX-M gene is found in 58.8% of P. aeruginosa isolates, while the KPC gene is not found in the isolates.*

Conclusion: *Our findings suggest that P. aeruginosa is one of the most common causes of DFU infection in Iraqi patients and The levels of MDR of P. aeruginosa isolates in Najaf hospitals were undoubtedly high. Most bacterial isolates show the presence of the CTX-M gene. The presence of these genes is one of the virulence factors and probably partly explains the resistance to antibiotics.*

Keywords: *Pseudomonas aeruginosa,* Diabetic foot ulcers (DFUs), CTX-M, and KPC genes.

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INTRODUCTION

Diabetes mellitus is a chronic but non-contagious metabolic disease characterized by elevated levels of blood sugar (glucose), absolute or relative insulin deficiency, or both, produced by the beta cells of the Islets of Langerhans within the pancreas. For the run-short of insulin, the body loses its ability to properly oxidize carbohydrates and to make the balance of sugar in the blood during energy production(Mahajan & Mohajan, 2023). There are multifaceted complications that account for more than 50% of these direct costs, such as nephropathy, neuropathy, retinopathy, atherosclerosis, and foot ulcers (Organization, 2016). These secondary pathophysiological outcomes are a result of a deficiency in the vascular system, causing inefficient circulation (Afonso *et al*., 2021). Among all of

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these diabetes complications, foot ulcers are at higher risk of occurring, and it is estimated that 20% of hospital admissions among DM patients result from diabetic foot ulcers (DFUs) (Afonso *et al*., 2021).

DFUs can be colonized by pathogenic bacteria and infection is favored by the immunological deficiencies related to diabetes (Baig *et al.*, 2022). When bacteria form biofilms, the cells are embedded in a self-produced polymeric matrix, which confers them protection from the host's immune system and antibiotics (Versey *et al.*, 2021). As a consequence, biofilms in DFUs may be responsible for the delayed healing and consequent infection chronicity, despite systemic antibiotic treatment(Price *et al*., 2020). One of the most common pathogens in chronic wounds is *Pseudomonas aeruginosa*, a very problematic microbe due to its ability to form resistant biofilms. *P. aeruginosa* is a ubiquitous and opportunistic pathogen with a prominent role in many infectious diseases, such as nosocomial urinary tract infections, cystic fibrosis infections, or chronic wounds, such as DFU infections (Barrigah-Benissan *et al.*, 2022). In this regard, (Srivastava & Sivashanmugam, 2020). found that most of the isolates in the DFU are *P. aeruginosa*(Mottola, 2017), and it has an inherent resistance to a broad spectrum of drugs(Mottola, 2017). Enzyme synthesis, efflux pump overexpression, porin deficiencies, and target-site changes have all been reported as resistance mechanisms in *P. aeruginosa* (Pang *et al*., 2019). Moreover, increased expression of AmpC βlactamase is frequently linked to β-lactamase resistance in *P. aeruginosa* (Gaibani *et al.*, 2022). In addition, cross-resistance may play a role in *P. aeruginosa's* multidrug resistance (Gaibani *et al.*, 2022). Indeed, *P. aeruginosa* is intrinsically resistant to several antimicrobials and can acquire mobile genetic elements (MGEs) due to the chromosomally encoded resistance genes (Botelho *et al*., 2019). This last mechanism is crucial in forming and disseminating drug resistance(Pang *et al*., 2019). Bacteria acquire genes to survive in unfavorable environments, namely when antibiotics are present (Pang *et al*., 2019). Plasmids and other MGEs are essential in this process since they serve as vectors for capturing and disseminating resistance genes. Exotoxin A, exoenzyme S, elastase, and sialidase are some virulence factors identified in *P. aeruginosa*. They are tightly controlled by cell-to-cell signaling networks (Keller & Surette, 2006). Hence, in the current study, we aim to detect the *blaCTX-M gene* and *blaKPC* gene genes in *P. aeruginosa* isolates from diabetic foot ulcers (DFUs) patients. The hypothesis is that these two genes are present in *P. aeruginosa* isolated from diabetic foot ulcers (DFUs) patients and they are part of the resistance system to antibacterial treatments.

MATERIALS AND METHODS

Patients and clinical specimens' collection

In this study, we have recruited 120 patients with DFUs whose range of age is (20-70 years) from both genders and over a specific time (September 2020 to January 2021). Patients were recruited from AlSadder Medical City and Al-Hakeem General Hospital in Najaf City, Iraq. Swabs were collected from all patients with a swap collection tube from JSHXRT company, China. In this study, infection and colonization were distinguished according to the Centers for Disease Control and Prevention (CDC, 2008) criteria. We followed the Iraqi and international ethics and privacy laws to perform the current study. Before participating in the present study, written informed consent is obtained from each individual. In the current study, the College of Medicine at the University of Kufa in Iraq has provided the IRB approval (617/2020)

compliant with the Declaration of Helsinki's International Guideline for Human Research Protection.

Isolation and identification of the bacterial isolates

After the samples were collected, we transferred them to the laboratory to inoculate on appropriate media, such as MacConkey's agar and blood agar plates and incubated them at 37°C for 18-24 hours. Gram staining, colony morphology on different media, and biochemical tests were used to make the primary diagnosis of isolates. One hundred twenty isolates were identified and validated using the Vitek2 system, which uses a kit (GP-ID cards) for Gram-positive bacteria and Gram-negative bacteria identified by (GN-ID cards) with ID message confidence levels ranging from very good to outstanding (probability percentage from 95 to 99). In addition, using the VITEK2-automated system, the final identification of bacteria isolates was confirmed biochemically. We examined the antibacterial resistance of *P. aeruginosa* isolates by the VITEK 2 automated system **Figure 1.** Susceptibility profiles were performed with 14 antibiotics in the specific AST cards, including ticarcillin with clavulanic acid, piperacillin, Ceftriaxone, Cefotaxime, Cefepime, Ceftazidime, Azithromycin, Levofloxacin, Gentamycin, Amikacin, Tobramycin, Ciprofloxacin, and Imipenem.

DNA Extraction

The sample preparation of DNA extraction was done as follows: a) one ml of the overnight bacterial growth on brain heart infusion (BHI) broth was taken and placed in a 1.5 ml microcentrifuge tube and centrifuged at 10000 rpm for 1 minute, b) the supernatant was thrown away, and the bacterial cell pellets were utilized to extract genomic DNA, c) genomic DNA was extracted according to the manufacturer's procedure using a commercial extraction kit (Genomic DNA Promega Kit). UV spectroscopy was used to assess the quality and quantity of DNA isolated from bacterial cultures. The Optical Density (OD) at 260/280 nm was used to evaluate DNA purity on a Shimadzu UV-1800 double-beam UV/Visible Scanning Spectrophotometer.

PCR amplification

As previously described (Barrigah-Benissan *et al.*, 2022), DNA samples (5 µL) were subjected to PCR apparatus in a 25µL reaction mixture using Sure Cycler 8800 (Agilent Technologies, Inc. USA). The following specific thermal cycling pattern was used to amplify the *blaCtxm* gene: 35 cycles consisting of 4 min initial denaturation, 30 seconds (s) at 94°C for denaturation, 63 S at 58°C for annealing, 1min at 72°C for extension, and 5 min for the final extension. *blaKp*c, gene amplification was performed following the following conditions: initial denaturation step at 94°C for 5 min; 35cycles of denaturation at 94°C for 60 s, annealing at 50°C for 60 s, extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min.

Table 1 lists the specific sequence primers used by Alpha DNA Company (Canada). Electrophoresis was used to analyze DNA fragments in a 1% agarose gel at 70 V for 1.5 h. in TBE 1X containing 4 µL of 10 mg/mL ethidium bromide using a 100-bp DNA ladder (Bioneer, Korea) as a size marker. On an ultraviolet light transilluminator (Cleaver, UK), a single band was seen at the appropriate spot; bands were photographed using a gel documentation system (Cleaver, UK).

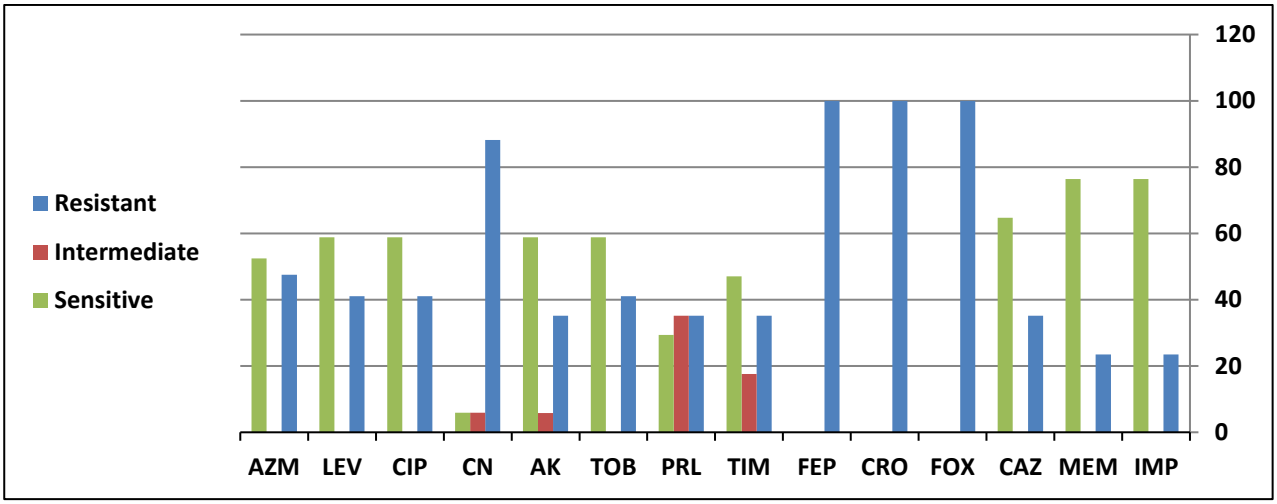

Figure 1: Susceptibility of isolates *P. aeruginosa* to antibiotics.

Table 1 : Primers used in this study.

Gene	Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extension
blaCtxm	94 Cº for 4min.	35	94 Cº for 30Sec	63Cº for 1 min	72 Cº for 1min.	72Cº for 5min.
blaKpc	94 Cº for 5 min.	35	94 Cº for 1min	50Cº for 1min.	72 Cº for 1 min	72Cº for 10min.

RESULTS

Distribution and characteristics of patients

Table 2 shows the patients' distribution and demographic characteristics across two hospitals that provided the samples. Most of the patients were recruited from AL-Sadar Medical City in Najaf province. We found that 86 (71.6 %) specimens were acquired from males and 34(28.3%) from females. Based on the residency, 29.2 % of specimens were taken from urban patients, whereas 70.8% of from rural. **Table 3** shows the patient distribution by age and gender. We found the most DFUs prevalent among older aged 51- 60 and the least prevalent among 20-30 from both genders. Table 4 revealed that most bacterial isolates in this study were Gram-negative more than Gram-positive.

Detection of the *blaKPC* Gene

The *KPC*gene of *P. aeruginosa* was detected in the isolates using the PCR technique, All *P. aeruginosa* isolates gave negative results with the *blaKPC* gene.

Detection of the *blaCTX-M gene*

The result showed that the blaCTX-M resistance gene was detected in 10/17(58.8%) *P. anginose* isolates as in **Figure 2.**

Table 2: Distribution and characteristics of patients recruited in the presented study.

Variables	AL-Hakeim Teaching Hospital	AL-Sadar Medical City
No.	40	80
Gender (M/F)	28/10	58/24
Residency (R/U)	19/21	32/48
Diagnosis (GP/GM)	40/10	60/10
No.: Number, M: Male, F: Female, R: Rural, U: Urban, GP: Gram-Positive, GM: Gram Negative.		

Table 3: Age-Gender distribution of the patients.

Gender	Age group No.					
	20-30	**31-40**	**41-50**	**51- 60**	**61-70**	**Total**
Female	1	6	10	17	0	34 (28.3%)
Male	3	19	28	34	2	86 (71.7%)
Total	4	25	38	51	2	120 (100%)

Table 4: Distribution of type of bacteria with the residence of patients.

Residence	Positive	Negative	Total %
Urban	5	30	35 (29.2%)
Rural	15	70	85 (70.8%)
Total	20	100	120 (100%)

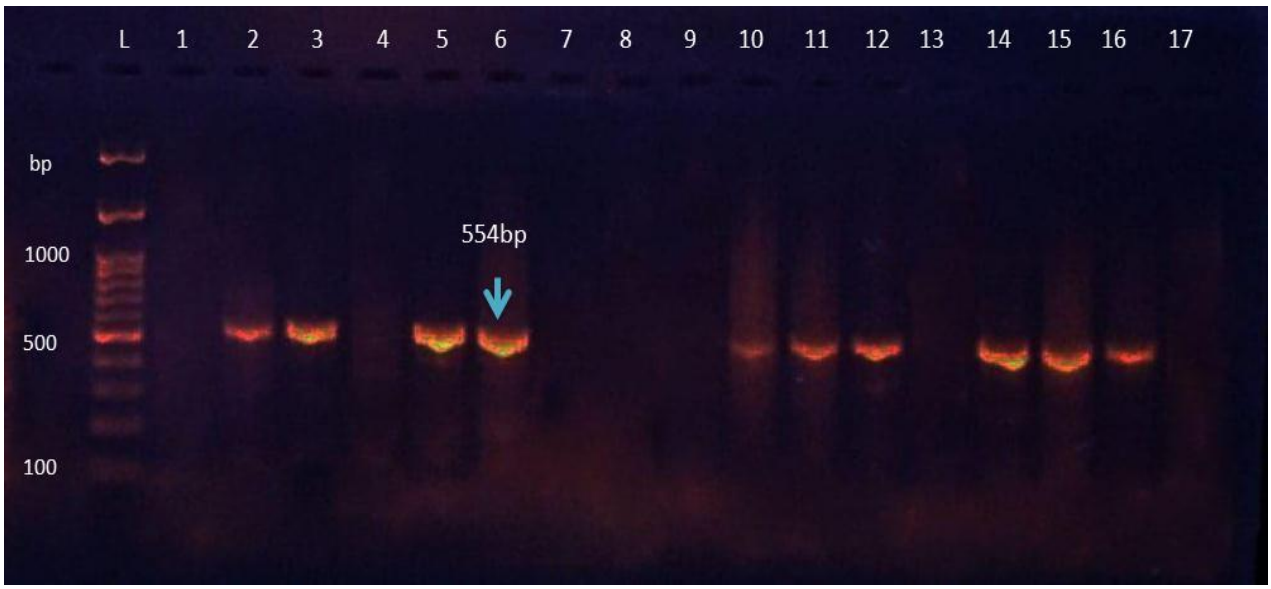

Figure 2: PCR amplification products of *P. aeruginosa* isolates that amplified with *blaCTX-M* gene primers with product 554 bp. Lane (L), DNA molecular size marker (100-bp ladder), and Lanes (2,3,5,6,10,11,12,13,15,16,17) show positive results with *blaCTX-M* gene.

DISCUSSION

P. aeruginosa isolates give negative results with the blaKPC gene. The blaKPC genes that encode KPCs are present on transferable plasmids and are flanked by transposable elements, thus allowing for the gene to move from the plasmid to the bacterial chromosome and back (Hu *et al.*, 2021). All the carbapenem-resistant isolates showed 100% resistance to ceftriaxone, cefoxitin, and Cefepime. The resistance to aminoglycoside antibiotics varied where tobramycin, ciprofloxacin, and levofloxacin with a percentage of (41.1%); Amikacin with a percentage (of 35.2%) Thus, blaKPC gene Detection in Clinical Isolates of Carbapenem-Resistant Enterobacteriaceae was MHT negative, This may have developed a different resistant mechanism other than carbapenemase production. Resistant to both imipenem and meropenem is a strong indicator of carbapenemase production rather than resistance to either one of the carbapenems, as this may imply a different resistance mechanism (Hassuna *et al.*, 2020).

The coexistence of antimicrobial resistance determinants, which virulence factors may partially explain in *P. aeruginosa* isolates, is a serious issue in treating this bacterial infection (Gaibani *et al.*, 2022). It is essential to detect all mediators in multidrug-resistant organisms to determine the optimal treatment options for such infections. The pathogenicity of *P. aeruginosa* was measured using a variety of cell-associated chemicals, including (e.g., flagella, pili, alginate capsule, and lipopolysaccharide) (Urgancı *et al.*, 2022). *P. aeruginosa*, on the other hand, can secrete substantial amounts of extracellular components (mainly toxins like exotoxin A and exoenzymes) as well as enzymes that may contribute to pathogenicity (e.g., proteases, elastases, phospholipase C, and neuraminidases)(Chadha *et al.*, 2022). Differences in virulence factor gene distributions in populations increase the likelihood that some *P. aeruginosa* strains are more adapted to the unique conditions encountered in specific infectious locations. Hence, virulence gene expression varies

according to the infection site and severity*(Jurado-Martín et al.*, 2021).

Another virulence factor recruited by *P. aeruginosa* is ESBLs are one of the main leading causes of resistance to ß-lactam antibiotics among Gram-negative bacteria. These enzymes are plasmid-encoded β-lactamases that mediate resistance to penicillins, first-, second-, and third-generation cephalosporins, such as cefotaxime, ceftriaxone, and ceftazidime. TEM, SHV, and CTX-M are the major genetic groups of ESBLs amongst clinically important Gram-negative bacteria (Klein, 2020). A higher frequency of these genes in isolates resistant to treatment indicates the involvement of these genes in drug resistance(Klein, 2020). In particular, *P. aeruginosa* is a gram-negative bacterium with high adaptability and intrinsic resistance to antibiotics, enabling it to colonize a variety of settings(Laborda *et al.*, 2021). *P. aeruginosa* can avoid the host immune response by minimizing blood flow and forming a shell that protects itself(Tian *et al.*, 2022). It has several exotoxins and enzymes that are secreted passively or through the secretion system; most importantly, exotoxin A (toxA), protease (lasA), and elastase (lasB), all these virulence factors secreted via secretion system type II (Mahmood, 2023). In Najaf city of Iraq, we found increased antibiotic resistance as the people can get any antibiotic over the counter, lack of hygiene, especially in government hospitals that enhances the prevalence of many opportunistic bacteria such as *P. aeruginosa* the potential of bacteria to adapt with various environments. There are some limitations in the current study that should be considered when interpreting the present findings. First, we could not examine the association of KPC and CTX-M genes with different antibiotic resistance. However, many of the isolates were resistant to some antibiotics. Further studies should examine only resistant bacteria. Moreover, Good infection control practices are combined with prudent antimicrobial policies could guarantee a limitation in the spread and expansion of MDR and XDR isolates , Investigating the nationwide occurrence of β-lactamases in clinical isolates of *P. aeruginosa* and real-time polymerase chain reaction (RT-PCR) should be recruited to determine whether these genes are translated and produce proteins.

CONCLUSION

Our findings suggest that *P. aeruginosa* is one of the most common causes of DFU infection in Iraqi patients and The levels of MDR of *P. aeruginosa* isolates in Najaf hospitals were undoubtedly high. Most bacterial isolates show the presence of the CTX-M gene. The presence of these genes is one of the virulence factors and probably partly explains the resistance to antibiotics.

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