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Clonal repetitive element polymerase chain reaction patterns of *Pseudomonas aeruginosa* in diabetic foot ulcers, Iran

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ABSTRACT

Objectives: *Pseudomonas aeruginosa* has gained attention in diabetic foot infections, which complicate treatment. Further research is essential to understand the prevalence and clinical impact of *P. aeruginosa* in diabetic foot ulcers (DFU) and to develop effective management strategies.

Methods: Samples were collected from 66 patients with DFU. The prevalence of *P. aeruginosa*, its antimicrobial profile, and biofilm formation were assessed by disk diffusion and crystal violet assays. The prevalence of resistance and virulence genes, including bla_{TEM}, bla_{SHV}, *tox*A, *alg*44, and *muc*A, was assessed using polymerase chain reaction. Finally, the clonality of the isolates was assessed by repetitive element polymerase chain reaction.

Results: The highest levels of resistance were seen against ciprofloxacin, tobramycin, and imipenem, with 58.6%, 57.1%, and 55.1%, respectively. A total of 41.3% and 62.5% of the isolates were strong biofilm-producers and multidrug-resistant, respectively. The prevalence of *tox*A, *alg*44, and *muc*A, were reported to be 82%,93.1%, and 75.8%, respectively, and for β-lactamase genes, such as bla_{TEM} and $bla_{SHV,}$ were 65.5% and 0%. Among the 28 isolates, 14 GTG types showed clonal relationships with certain strains.

Conclusion: These findings suggest that all clonal types were associated with the same hospital, emphasizing the need for epidemiologic surveillance of hygiene practices within healthcare facilities to mitigate strain dissemination.

Introduction

Diabetes has become a significant public health concern, markedly increasing the incidence of chronic metabolic diseases and related mortalities. Individuals with diabetes have an increased risk of severe complications, leading to increased medical costs, diminished quality of life, and higher mortality rates [1,2]. According to the International Diabetes Federation, the number of adults aged 20 years and older living with diabetes is projected to increase from 463 million in 2019 to 700 million by 2045 [3].

Among the various complications of diabetes, diabetic foot ulcers (DFUs) are particularly prevalent, affecting approximately 6.3% of patients worldwide [4]. The healing or non-healing of these ulcers is significantly influenced by the microbial communities in the wound and their pathogenic potential. A validated classification system for foot ulcers is essential for clinicians to effectively manage diabetic foot problems, making accurate identification of causative pathogens crucial for appropriate treatment [5].

Diabetic foot infections (DFIs) are characterized by a diverse array of pathogens, including aerobic gram-positive cocci, aerobic gram-negative bacilli, and anaerobic organisms that can form biofilms in wound environments. The ability of these microorganisms to create biofilms is a critical virulence factor that enables them to survive and resist antibiotic treatments [6]. Biofilms are a major contributor to chronic infections, such as DFUs, and are linked to the increase of multidrug-resistant (MDR) strains, resulting in frequent treatment failures. Despite regular antibiotic therapy, the lack of a routine assessment of biofilm formation often leads to unsuccessful outcomes in patients with diabetic ulcer patients [7].

Common pathogens isolated from DFIs include gram-positive organisms, such as *Staphylococcus aureus* and *Enterococcus*, and gram-negative bacteria, such as *Pseudomonas aeruginosa, Escherichia coli, Klebsiella* species, and *Proteus* species, many of which exhibit multidrug resistance. The increasing prevalence of antibiotic-resistant gram-negative infections, particularly, DFIs, presents a significant public health challenge [8].

P. aeruginosa has drawn considerable attention owing to its association with specific DFIs, particularly, in cases involving puncture wounds, osteomyelitis, and the use of moist wound dressings. However, the actual prevalence of *P. aeruginosa* in DFIs remains unclear. Some studies

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have identified it as a common isolate, whereas others have suggested that it may not be as predominant as previously assumed. This inconsistency underscores the need for further research on the epidemiology and role of *P. aeruginosa* in DFIs [9].

Moreover, the increasing incidence of MDR *P. aeruginosa* infections, particularly, among hospitalized patients, raises concerns regarding antimicrobial resistance. Resistance is often exacerbated by the transmission of resistant strains and the acquisition of new resistance mechanisms due to previous antibiotic exposure [10]. Recognizing the presence of MDR or extensively drug-resistant *P. aeruginosa* is vital for selecting appropriate treatments for severe systemic infections and ensuring timely and effective therapy.

The novelty of this study is that it addressed the gaps in our understanding of the true prevalence and clinical significance of *P. aeruginosa* in DFUs. We aimed to analyze the bacterial profiles associated with DFUs and evaluate the antimicrobial susceptibility patterns of the isolates. In addition, we aimed to investigate the clonality of the isolates to determine whether they originate from a single clone and investigate the circulation of these isolates within hospital settings to trace their origins. By focusing on these aspects, this study sought to provide insights into effective treatment strategies and ultimately improve patient outcomes.

Materials and method

Sample collection and bacterial isolation from the diabetic foot ulcers

This cross-sectional study included 66 adult patients with diabetes mellitus who presented with active foot ulcers. The local ethics committee of Isfahan University of Medical Sciences approved this study (IR. MUI. MED. REC.1401.405). The participants provided written informed consent to participate in this study.

Active foot ulcers were defined as non-healing wounds identified during a comprehensive physical examination and considered more likely to be infected. Microbiological samples were obtained from the deepest parts of the foot ulcers using the deep-swab technique [11]. Two sterile swabs were soaked in sterile glucose broth and immediately transported to the microbiology laboratory at Isfahan University of Medical Sciences, with a maximum transit time of 2 hours. The collected samples were cultured on blood agar and MacConkey agar and catalase, oxidase, IMViC, and oxidative-fermentative tests are a few examples of conventional biochemical tests that were performed; and *P. aeruginosa*–suspected colonies were recultured to achieve a pure and single colony and production of the blue-green pigment pyocyanin on cetrimide agar and growth at 42°C [12,13].

Antibiotic susceptibility testing

Antimicrobial resistance profiles of the isolated pathogens were determined using the Kirby-Bauer disk diffusion method according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI 2023) [14]. The bacterial isolates were tested against a panel of the following antibiotics: ciprofloxacin (5 μg), piperacillin-tazobactam (30 μg), piperacillin (100 μg), ceftazidime (30 μg), tobramycin (10 μg), cefepime (30 μg), and imipenem (10 μg). The inoculated plates were incubated at 37°C for 18-24 hours. The zones of inhibition were measured, and the results were interpreted according to the CLSI break point criteria (CLSI 2023) to categorize the isolates as susceptible, intermediate, or resistant to the tested antimicrobials. Furthermore, the isolates were classified as MDR if they demonstrated resistance to at least one agent in three or more antimicrobial classes, as defined by the criteria established by Magiorakos *et al.* [15].

Biofilm formation assay

The ability of bacterial isolates to form biofilms was evaluated using the standard plastic microtiter plate method. Briefly, 230 μl of Tryptic case Soy Broth was added to each well of a sterile 96-well flat-bottomed polystyrene microplate. Then, 20 μl of an overnight bacterial culture was inoculated into the appropriate wells (in triplicates for each strain).

The microplates were incubated aerobically at 35°C for 24 hours. After the incubation period, the well contents were gently discarded, and the wells were washed three times with 200 μl of sterile phosphate buffer saline to remove any non-adherent bacteria. Adherent bacteria were fixed with 200 μl of ethanol for 15 minutes, followed by staining with 200 μl of 0.1% crystal violet solution for 10 minutes. The excess stain was removed by washing five times, and the plates were air-dried.

The dye bound to the adherent bacteria was solubilized by adding 200 μl of 33% (v/v) glacial acetic acid to each well. Each well's optical density (OD) was measured at 570 nm using an enzyme-linked immunosorbent assay plate reader [15]. The experiment was performed in triplicate, and the results were averaged. The cut-off OD value was defined as three SDs above the mean OD of the negative control wells. The strains were classified as biofilm-producers or non–biofilm-producers based on the established criteria [16]. *Escherichia coli* K12 and *Pseudomonas aeruginosa* ATCC 27853 were used as the negative control (weakly biofilm-forming) and positive control (strong biofilm-forming) strains, respectively.

Table 1
Sequences of primers used in this study.

Gene	Primer sequence (5′-3′)	Product (bp)	References
*muc*A	F: CGC GAG TTC GAA GGT TTG AG R: CAC GCG ATG CCA GAT CTC AT	942	This study
*tox*A	F:GAC AAC GCC CTC AGC ATC AC R: CGC TGG CCC ATT CGC TCC AG	397	[18]
*alg*44	F: TCT ACG TGT TCT TCC GCC TC R: TTG AGC ATG TCC AGC AGG TT	959	This study
bla_{TEM}	F: AGT ATT CAA CAT TTC CGT GTC R: GCT TAA TCA GTG AGG CAC CTA TC	850	[19]
bla_{SHV}	F: ATG CGT TAT ATT CGC CTG TG R: GTT AGC GTT GCC AGT GCT CG	862	[19]
Rep-typing	GTG GTG GTG GTG GTG	Variable	[19]

PCR amplification of bla_{TEM}, bla_{SHV}, toxA, alg44, and mucA genes

Bacterial genomic DNA was extracted using the phenol-chloroform method, as described by Sambrook and Russell [17]. The purity and quality of the extracted DNA were evaluated using a NanoDrop spectrophotometer, and samples with an OD260/OD280 nm ratio ≥1.8 were considered suitable for further analysis. Polymerase chain reaction (PCR) was performed to detect the presence of various resistance and virulence genes, including bla_{TEM}, bla_{SHV}, *tox*A, *alg*44, and *muc*A. The specific primer pairs used for each target gene are listed in Table 1. PCR was performed according to standard protocols, and the amplified products were analyzed by agarose gel electrophoresis [20]. The DNA of the positive controls was obtained from *E. coli* isolates that were sequenced from Iranian patients who underwent kidney transplant for bla_{TEM} and bla_{SHV}; we used the sequenced DNA of *P. aeruginosa* that was positive for virulence genes.

(GTG)5-Repetitive element PCR (Rep-PCR) fingerprinting technique

The genetic diversity of the bacterial isolates was evaluated using the (GTG)5-PCR fingerprinting technique, as described by Gevers *et al.* [21]. The (GTG)5 primer (5′-GTGGTGGTGGTGGTG-3′) was used to amplify the repetitive sequences present in the chromosomal DNA of the bacterial isolates. The PCR conditions were as follows: initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 5 minutes. The amplified products were separated by electrophoresis on a 1.5% agarose gel in 0.5× Tris-borate-ethylenediamine-tetraacetic acid buffer

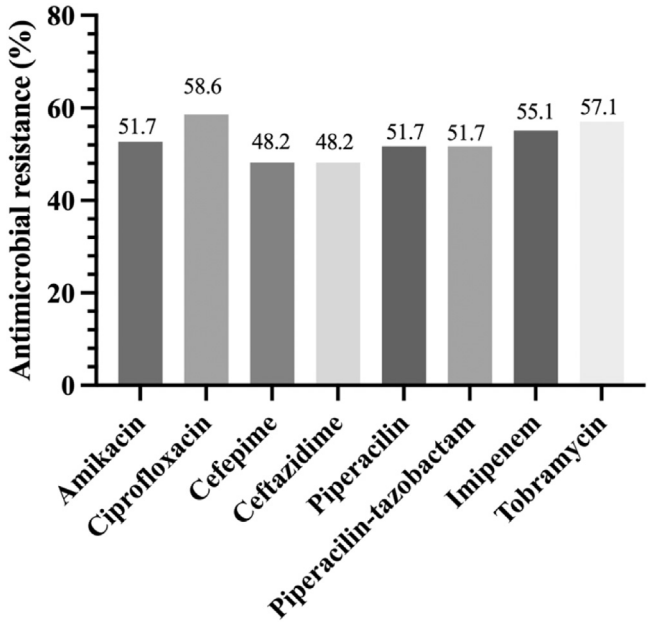

Figure 1. Frequency of antimicrobial resistance of *P. aeruginosa* strains.

at 65 volts for 2.5 hours. The band patterns were visualized using a 50 bp DNA ladder as a reference. The (GTG)5-PCR fingerprint patterns were analyzed using the curve-based algorithm (Pearson correlation) and the unweighted pair group method with arithmetic mean for cluster analysis, as implemented in the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The cut-off value for defining strain relatedness was set to 100% similarity [22]. This experiment was performed in triplicate to ensure reproducibility of the results.

Discriminatory power of the (GTG)5-PCR fingerprinting technique

To assess the discriminatory ability of the (GTG)5-PCR fingerprinting technique, we calculated Simpson's diversity index, also known as the discriminatory index (D). This index provides a measure of the probability that two randomly selected, unrelated strains from the test population will be classified into different typing groups.

The D was calculated using the following formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} xj(xj-1)$$

N is the total number of strains evaluated, S is the number of distinct types identified, and xj is the number of strains belonging to the jth type (each type).

Statistical analysis

Genetic diversity among the bacterial isolates was evaluated using Nei's distance measure, as described by Weir [23]. This approach allowed the quantification of genetic distances between isolates based on genotypic fingerprinting data. As reported in previous studies, Simpson's diversity index was calculated to assess the discriminatory ability of rep-fingerprinting methods [24]. This index provides a measure of the probability that two randomly selected, unrelated strains will be classified into different typing groups. Statistical analyses were performed using IBM SPSS Statistics for (version 16). Depending on the data distribution and sample size, the chi-square test or Fisher's exact test was

Figure 2. Dendrogram showing genetic relatedness of 28 strains of *P. aeruginosa* determined by rep-polymerase chain reaction analysis with Dice similarity coefficient and unweighted pair-group method with average linkages clustering method. Cut-off value: 100%.

Table 2
The severity of biofilm and bla_{TEM}, bla_{SHV}, *tox*A, *muc*A, and *alg*44 prevalence.

Biofilm	bla_{TEM} NO. (%)	*tox*A NO. (%)	*alg* 44 NO. (%)	*muc A* NO. (%)	bla_{SHV} NO. (%)	Total NO. (%)
Strong	5 (45.4)	9 (81.8)	9 (81.8)	7(63.6)	0 (0)	12 (41.3)
Moderate	12(73.3)	13(8.6)	15 (100)	14(93.3)	0 (0)	15 (51.7)
Weak	2 (100)	1(50)	2 (100)	1 (50)	0 (0)	2 (10.34)
Total	19 (65.5)	23	26 (89.6)	22 (75.8)	0	29 (100)

used to determine any statistically significant associations between the variables. A P <0.05 was considered statistically significant, indicating a low probability that the observed differences or associations were due to chance.

Results

Sample collection and bacterial isolation from the diabetic foot ulcers

Of the 66 DFU samples examined (41 men and 25 women), 42% (28) were positive for *P. aeruginosa*. In this study, 28.6% (eight) of *P. aeruginosa* isolates were from females and 71.4% (20) were from males. The median age was 65.5 years (17-90).

Antibiotic susceptibility testing

As shown in Figure 1, the isolates displayed the highest levels of resistance to ciprofloxacin, tobramycin, and imipenem (58.6%, 57.1%, and 55.1%, respectively). The highest susceptibility was observed for ceftazidime and cefepime, with 48.2% and 44.8% of isolates showing susceptibility to these antibiotics, respectively. Of 28 *P. aeruginosa* isolates, 62.5% were MDR.

Biofilm formation assay

All 28 *P. aeruginosa* isolates obtained from DFUs were capable of forming biofilms. Among the 28 *P. aeruginosa* isolates, 12 (41.3%) exhibited strong biofilm-forming ability (OD590 nm ≥2.5), 15 isolates (54%) were classified as moderate biofilm-producers, (1.5 ≤ OD590 nm < 2.5), and one isolate (7%) was identified as weak biofilm former, (0.7 ≤ OD590 nm < 1.5).

Polymerase chain reaction amplification of bla_{TEM}, bla_{SHV}, toxA, alg44, *and* mucA genes

The prevalence rates of virulence genes, including *tox*A, *alg*44, and *muc*A, were 82%,93.1%, and 75.8%, respectively. Also, the prevalence of β-lactamase genes, including bla_{TEM} and bla_{SHV}, were reported to be 65.5% and 0%, respectively. There was no significant relationship between the presence of surveyed genes with biofilm severity and MDR (P >0.05) (Table 2).

(GTG)5-Rep-PCR fingerprinting technique

Repetitive element (rep)-PCR or (GTG)5-PCR fingerprints of the 28 isolates generated 2-8 bands, with molecular sizes ranging from 200 bp to 500 bp. Of the 28 isolates, 14 GTG types were identified with a cut-off value of 100%, and five isolates had a single type (Figure 2). D was calculated from a constructed phylogenetic tree, and, according to the formula described previously, the discriminatory power was estimated at 94%.

Discussion and conclusion

DFUs represent a significant and costly complications in individuals with type 2 diabetes. Projections indicate that the incidence of DFUs in patients with diabetes may continue to increase annually, potentially reaching 50% [25,26]. MDR bacteria are the prevalent pathogens in DFUs and can exacerbate these conditions. The reasonable use of antibiotics is crucial for enhancing treatment outcomes in DFIs, minimizing adverse effects, and curtailing the development of bacterial resistance [27,28]. Although DFUs are inherently polymicrobial and multifactorial, this study focused on the prevalence of *P. aeruginosa* because of ongoing debates regarding its role in such infections. The incidence and risk factors associated with this microorganism in DFUs have been discussed extensively, although the existing evidence remains limited or inconsistent [18]. *P. aeruginosa* is a widespread and opportunistic pathogen that is implicated in various infectious diseases, including nosocomial urinary tract infections, cystic fibrosis, and chronic wounds such as DFUs [29]. Furthermore, investigating the prevalence of MDR *P. aeruginosa* strains among patients with DFUs is vital because these bacteria can transfer resistance genes to other microorganisms via mobile genetic elements such as transposons, integrons, and plasmids [27]. The level of antibiotic resistance varies by country and is influenced by prescribing practices, use of foreign medical devices, and health care facility standards [30]. In this study, the prevalence of *P. aeruginosa* among patients with DFU was 43%, which is notably higher than that reported in other studies conducted in Iran [31,32]. Other studies have indicated that the prevalence of *P. aeruginosa* in DFIs across Asia ranges from 1.63% to 100%, whereas in western countries, the prevalence ranges from 0.5% to 50%, with most studies reporting rates below 20%. In Africa, the prevalence varies from 9.4% to 32.2% [31]. Some studies have reported that *P. aeruginosa* accounts for up to 40-50% of DFIs, which is consistent with our findings [33–35]. In our study, 62% of patients with DFUs were male, corroborating other studies that indicated a male predominance of foot ulcers and related complications [32,36,37]. This may be attributed to more severe neuropathy, reduced joint mobility, and increased foot pressure in male patients with diabetes [38]. This study identified the main gram-positive species in the DFIs as *S. aureus* (9.09%) and *Micrococcus* (1.5%). The predominant gram-negative species included *E. coli* (9.09%), *Klebsiella pneumoniae* (15%), *Proteus mirabilis* (3.03%), *P. aeruginosa* (43%), and *Acinetobacter baumannii* (4.5%), which is consistent with the findings of previous studies [32,35].

Our antimicrobial susceptibility results indicate that ciprofloxacin, tobramycin, and imipenem exhibited the highest resistance, in contrast to the findings of Yakout and Abdelwahab [35]. This discrepancy could be due to differences in prescription patterns, geographical variations, and the year of isolation. In addition, our study identified ceftazidime and cefepime as effective antibiotics, which is consistent with the results of other researches [19,35]. These data suggest that the prevalence of *P. aeruginosa*, particularly, the MDR strains, in diabetic foot ulcer infections is relatively high, with a corresponding high level of inherent antibiotic resistance, which can lead to delayed wound healing. Therefore, prompt treatment is essential [18]. The findings of this study agree with those of earlier studies indicating that 69.7% of *P. aeruginosa* isolates were MDR [30,35], in contrast to some studies that reported lower rates of MDR isolates [39,40].

Various studies conducted worldwide have reported an increased incidence of MDR bacteria. This increase is largely due to the widespread use of antibiotics, which creates selective pressure that favors the survival of pathogenic bacterial strains [41]. MDR *P. aeruginosa*, which is shielded by biofilms that are difficult to penetrate, can endure and acquire additional resistance [42]. *Pseudomonas* spp. is frequently found in immunocompromised individuals, which is attributed to their significant pathogenic potential and various virulence factors, particularly, in biofilm formation. These virulence factors and biofilm development are critical elements in the pathophysiology of DFUs. The establishment of bacterial biofilms is fundamental, providing protection against various environmental challenges, enhancing persistence in medical devices, facilitating evasion of the immune response, and contributing to the emergence of antimicrobial resistance [43].

Ertugrul *et al.* [44] showed that the prevalence of *tox*A virulence genes in *P. aeruginosa* isolated from DFUs was more significant than that in other kinds of samples and was 100% in line with our study that this prevalence was reported to be 82% [18].

The *alg*44 gene is one of the genes that control alginate production in *P. aeruginosa* bacteria and is one of the main virulence factors causing chronic infections in the human body; 93.1% of isolates in this study were positive for this gene, which is in line with the study by Wahyudi *et al.* [45] in 2022.

Although alginate is not essential for biofilm formation, it plays a vital role in biofilm development and architecture in *P. aeruginosa*, whereas most isolates can form biofilm that is correlated with the high prevalence of the alginate and *muc*A genes. MucA is an anti-sigma factor to the alternative sigma factor AlgU (also known as AlgT, σE, or σ22), which responds to envelope stress. MucA acts as an inhibitor of the sigma factor AlgU. Mutations in the *muc*A gene can cause AlgU misregulation, leading to a mucoid phenotype associated with adverse outcomes in cystic fibrosis [46].

Although the mutability of *muc*A suggests that it may be non-essential for bacterial survival, many researchers have demonstrated that, paradoxically, a segment of *muc*A is critical for the viability of *P. aeruginosa* [47]. The presence of multiple β-lactamase-producing *P. aeruginosa* strains can lead to substantial treatment failure and present serious clinical challenges if they are not identified. The current study results showed an increasing rate of β-lactamase resistance gene of bla_{TEM} compared with other studies [47,48]. Because the $bla_{\text{TEM-1}}$ gene is the most frequently detected plasmid-borne antimicrobial resistance gene, this might be because of the transfer of resistance genes through plasmids or other mobile elements and the circulation of this resistance gene among the strains in hospitals. In addition, some studies reported the rate of bla_{SHV} similar to our results [48,49], but some of them showed differences [50–52]. Although bla_{SHV} is a core chromosomal gene, it could be because of the source of samples and the difference in geographical area and the year of isolation. To identify the source of infection and prevent the spread of strains with similar clonality, it is essential to evaluate the types of the isolates. In our study of the 28 isolates, we identified 14 GTG types, indicating clonal relationships among certain strains. This clonality may have resulted from the transmission of isolates within the hospital environment, which is potentially facilitated by health care personnel or nosocomial infections [42]. The other isolates were categorized into distinct GTG types, highlighting their differences from other species. These findings suggest that all clonal types are associated with the same hospital, emphasizing the need for epidemiologic surveillance of hygiene practices within health care facilities to mitigate strain dissemination.

Declarations of competing interest

The authors have no competing interests to declare.

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Author contributions

NG: writing, review and editing, visualization, investigation. AM: writing – review and editing, writing – original draft, visualization, investigation. BNE: review, editing, and advising. SM: writing – review and editing, writing – original draft, supervision, resources, project administration, methodology, funding acquisition, and conceptualization.

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