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Original article

Plasmid-Mediated Resistance and Biofilm Formation in Gram-Negative Diabetic Foot Ulcer Infections

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Abstract
Diabetic foot ulcers (DFUs) are a significant healthcare challenge, particularly due to the increasing prevalence of multidrug-resistant (MDR) Gram-negative bacteria. This study aims to analyze the antibiotic resistance profiles, biofilm formation, and the role of plasmids in mediating resistance in bacteria isolated from DFU infections. A total of 27 patients with DFUs were enrolled, and bacterial samples were collected from both surface and deep tissue. Isolates were cultured, identified, and tested for antibiotic resistance using the disk diffusion method. The results showed high resistance rates, with Enterobacter cloacae and Klebsiella pneumoniae being the most predominant pathogens, each exhibiting 100% resistance to ceftazidime and amoxicillin-clavulanic acid. Notably, resistance to fluoroquinolones was also significant, with E. cloacae showing 44.44% resistance to levofloxacin. Extended-spectrum beta-lactamase (ESBL) production was also prevalent, particularly in Enterobacter cloacae and Klebsiella pneumoniae. Biofilm formation, which contributes to chronic persistence of infection, was also significantly higher in Enterobacter cloacae and Klebsiella pneumoniae. A significant correlation was observed between plasmid presence and increased antibiotic resistance, especially against tetracycline and fluoroquinolones, indicating the role of plasmids in resistance dissemination. These findings underscore the importance of plasmid-mediated resistance in DFU infections and highlight the need for local resistance surveillance and targeted treatment strategies. Future studies should incorporate molecular techniques, such as whole-genome sequencing, to further elucidate the genetic basis of resistance and biofilm formation in DFUs.
Keywords. Diabetic Foot Ulcers (DFUS), Antibiotic Resistance, Gram-Negative Bacteria, Biofilm Formation, Plasmid-Mediated Resistance.

Introduction

Diabetic foot ulcers (DFUs) pose a major global health challenge for people with diabetes, characterized by high prevalence and complex management. Bacterial infections frequently complicate these chronic wounds, increasing the risk of limb amputation and sepsis. Diabetics face a 25% lifetime risk of developing a DFU and a 15-46 times greater risk of amputation (1). Effective management of DFU necessitates a comprehensive understanding of the bacterial landscape, including the prevalence, resistance patterns, and virulence factors of the infecting microorganisms (2,3).

A major challenge in the treatment of diabetic foot ulcers (DFUs) is the increasing antibiotic resistance observed in gram-negative bacteria (4). Resistance mechanisms, including Extended-Spectrum Beta-Lactamases (ESBLs), AmpC beta-lactamases, Metallo Beta-Lactamases (MBLs), and Carbapenemases, significantly diminution the effectiveness of commonly used antibiotics, jeopardizing treatment success and patient outcomes (5). Plasmid-mediated resistance involves the transfer of genetic material that encodes resistance genes, which can spread rapidly among bacterial populations (6). Plasmids, which are small self-replicating DNA molecules, play a crucial role in the dissemination of resistance genes, thereby to the persistence of multidrug-resistant bacterial infections (7). While molecular techniques such as polymerase chain reaction (PCR) and sequencing are ideal for plasmid detection (8), they are often unavailable in resource-limited settings. In such a case, methods including plasmid profiling and conjugation experiments may offer alternatives (9, 10).

Biofilm formation and bacterial virulence factors further complicate treatment by enhancing resistance to antimicrobial therapy and evading the host immune response. Biofilms are complex communities of microorganisms that adhere to surfaces and are encased in a protective extracellular matrix, which makes them resistant to antimicrobial agents and host immune responses. This resistance fosters chronic infections and reduces treatment efficacy (11, 12). Studies have shown that biofilm formation, combined with virulence factors such as enzymes and immune-evasive mechanisms, plays a central role in the persistence of DFU infections (13, 14). While research has examined antibiotic resistance and biofilm formation in DFU-associated gram-negative bacteria, the interplay between plasmid-mediated resistance and biofilm development remains poorly understood, and further exploration is needed to clarify the virulence characteristics influencing bacterial invasiveness and resistance to therapy (15, 16).

The spread of multidrug-resistant bacteria, including carbapenemase-resistant strains, in Libya is exacerbated by inadequate hospital surveillance and infection control, including hygiene protocols. This increases treatment failure and worsens patient outcomes (17). Diabetic foot ulcers (DFUs) in Libya are

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frequently colonized by diverse, often mixed-species microbial populations that promote chronic wound progression. Biofilm formation and bacterial virulence further hinder treatment by fostering antimicrobial resistance and immune evasion (18). Insufficient surveillance programs fail to comprehensively track infection prevalence and patterns. The lack of effective infection control committees in Libyan hospitals reveals a systemic inability to prevent and control antibiotic-resistant bacteria (17).

This research investigates Gram-negative bacteria isolated from diabetic foot ulcers (DFUs), focusing on their prevalence, antibiotic resistance profiles, biofilm formation capabilities, and the role of plasmids in mediating antibiotic resistance. By characterizing plasmid-mediated resistance, the study seeks to inform the development of targeted DFU treatments, ultimately improving clinical outcomes and reducing infection burden.

Methods

Sample Collection and Cultivation

A cross-sectional study was conducted at the Diabetes and Endocrinology Center in Sebha City, enrolling 27 patients with diabetic foot ulcers. Ethical approval and informed consent (LY025415-12) were obtained. Swabs and deep tissue samples collected aseptically during debridement to minimize contamination were transported refrigerated to the laboratory. Samples were cultured on CLED and MacConkey agar at 37°C for 24–48 hours. Bacterial isolates were identified by culture characteristics and confirmed using API20E system.

Antibiotic Susceptibility Testing

Bacterial suspensions standardized to a 0.5 McFarland were inoculated onto Mueller-Hinton agar following CLSI guidelines (19). Disk diffusion susceptibility testing was performed using tetracycline (TE10), gentamicin (CN10), ciprofloxacin (CIP5), ceftolozane (CTI10), levofloxacin (LEV5), imipenem (IMP), ceftazidime (CAZ), cefotaxime (CTX), amoxicillin-clavulanic acid (AMC30), ceftriaxone (CRO), and cefoxitin (FOX). After 16-18 hours of incubation at 35°C, inhibition zone diameters were measured and interpreted according to CLSI criteria. Duplicate tests were conducted, and resistance percentages were calculated.

Phenotypic Screening for ESBL, AmpC, and MBL Production

ESBL production was assessed using the double-disk synergy test (DDST) as described by (20), Bacterial suspensions (0.5 McFarland standard) were incubated on Mueller-Hinton agar plates with third-generation cephalosporins (ceftazidime, cefotaxime, or cefpodoxime) and amoxicillin/clavulanic acid (20/10 µg) placed 20 mm apart. ESBL production was indicated by a synergistic effect (enhanced zone of inhibition between the cephalosporin and amoxicillin/clavulanic acid discs) after 16–18 hours at 35°C. AmpC β-lactamase production was screened by cefoxitin resistance; isolates exhibiting inhibition zones <18 mm around a 30 µg cefoxitin disk were further assessed using the boric acid-EDTA disk test (21). MBL production was detected using EDTA-imipenem discs. Two imipenem discs (10 µg) were placed on the agar, one with and one without 10 µL of 0.5 M EDTA. An increase of ≥7 mm in the inhibition zone surrounding the EDTA-supplemented imipenem disc compared to the control disc after 16–18 hours at 35°C indicated MBL production (22). EDTA inactivates MBLs by chelating zinc ions, thereby restoring imipenem susceptibility.

Biofilm Formation Assay

Biofilm formation was quantified using the crystal violet staining method (23). Bacterial isolates were cultured in tryptone soy broth (TSB) at 37°C for 18 hours to allow biofilm development. Following incubation, non-adherent cells were removed by washing with phosphate-buffered saline (PBS). Adherent biofilms were then stained with 0.1% crystal violet, which binds to the biofilm matrix. Excess stain was removed, and bound crystal violet was solubilized with 33% glacial acetic acid. Absorbance of the solubilized dye, correlating with biofilm biomass, was measured spectrophotometrically at 595 nm. Assays were performed in triplicate, and averaged results ensured reproducibility and accuracy (24).

Plasmid Isolation

Plasmids were extracted using the rapid boiling method (25). Bacterial cultures were grown in LB broth with amoxicillin (10 µg/mL) at 37°C for 24 hours (26). Plasmid DNA was visualized by agarose gel electrophoresis (0.8% Gel Red-agarose in 1X TAE buffer, 80V), and images were analyzed using ImageJ software.

Statistical Analysis

Statistical analysis was performed using Minitab (22). Pearson correlation coefficients assessed the relationship between plasmid presence and antibiotic resistance. One-way ANOVA evaluated differences in biofilm production, with statistical significance set at $p < 0.05$.

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Results

Distribution of Gram-Negative Bacteria in Diabetic Foot Ulcer Infections

The distribution of Gram-negative bacteria isolated from diabetic foot ulcer infections is summarized in Figure 1. Enterobacter cloacae was the most frequently identified bacterium, comprising 47.6% of isolates, followed by Klebsiella pneumoniae (35.7%). Pseudomonas spp. and Escherichia coli were less prevalent, each accounting for 8.3% of cases.

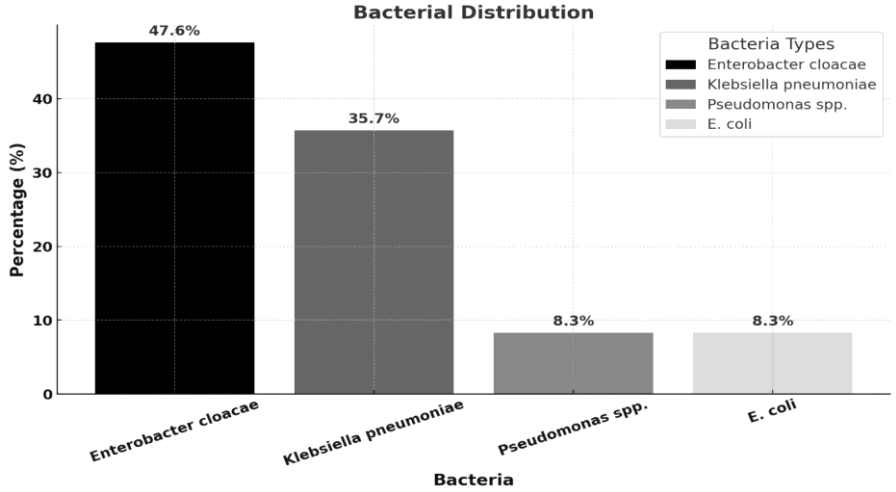

Figure 1. Illustrates the prevalence (%) of various Gram-negative bacteria in diabetic foot ulcer infections

Susceptibility Patterns of Gram-Negative Bacteria in Diabetic Foot Ulcers

Antibiotic susceptibility testing revealed notable resistance trends among the isolated Gram-negative bacteria. Table 1 E. coli and E. cloacae exhibited 100% resistance to Ceftazidime (CAZ) and Amoxicillin-Clavulanic Acid (AMC30). However, both species showed complete sensitivity to all other tested antibiotics, except Levofloxacin (LEV5) in E. cloacae, where 44.44% of strains were resistant. Klebsiella pneumoniae demonstrated 100% resistance to CAZ and AMC30, along with high resistance to Ceftriaxone (CRO) (85.71%), while showing mostly sensitivity to other antibiotics. Notably, 14.29% of strains exhibited resistance to Ceftolozane (CTI10). Pseudomonas spp. followed a distinct pattern, displaying 100% resistance to CAZ and AMC30, 33.33% resistance to Tetracycline (TE10), but complete sensitivity to Ciprofloxacin (CIP5), Ceftriaxone (CRO), and Ceftolozane (CTI10).

Table 1. Antibiotic Susceptibility Patterns of Gram-Negative Bacteria Isolated from Diabetic Foot Ulcers

Antibiotic	**E. coli**		**Enterobacter cloacae**		**Klebsiella pneumonia**		**Pseudomonas spp.**	
CAZ	R	S	R	S	R	S	R	S
	100%	0%	100%	0%	100%	0%	100%	0%
AMC30	R	S	R	S	R	S	R	S
	100%	0%	100%	0%	100	0%	100%	0%
TE10	R	S	R	S	R	S	R	S
	0%	100%	0%	100%	0%	100%	33.33%	66.67%
CN10	R	S	R	S	R	S	R	S
	0%	100%	0%	100%	0%	100%	0%	100%
CIP5	R	S	R	S	R	S	R	S
	0%	100%	0%	100%	0%	0%	0%	100%
CTI10	R	S	R	S	R	S	R	S
	0%	100%	0%	100%	14.29%	85.71%	0%	100%
LEV5	R	S	R	S	R	S	R	S
	0%	100%	44.44%	55.56%	0%	100%	0%	100%
IMP	R	S	R	S	R	S	R	S
	0%	100%	0%	100%	0%	100%	0%	100%
CTX	R	S	R	S	R	S	R	S
	0%	100%	0%	100%	0%	100%	0%	100%
CRO	R	S	R	S	R	S	R	S
	0%	100%	0%	100%	85.71%	4.29%	0%	100%

R = Resistant, S = Susceptible CAZ = Ceftazidime, AMC30 = Amoxicillin-Clavulanic Acid, TE10 = Tetracycline, CN10 = Gentamicin, CIP5 = Ciprofloxacin, CTI10 = Ceftolozane, LEV5 = Levofloxacin, IMP = Imipenem, CTX = Cefotaxime, CRO = Ceftriaxone.

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Prevalence of ESBL, AmpC, MBL, and Carbapenemase Production in Gram-Negative Bacteria

A total of 22 Gram-negative bacterial isolates were analyzed for beta-lactamase production, including Enterobacter cloacae (9), Klebsiella pneumoniae (7), Pseudomonas spp. (3), and E. coli (3). The results revealed significant variations in resistance mechanisms across species (Table 1). ESBL production was detected in 81.8% (18/22) of isolates, making it the most prevalent resistance mechanism. All Enterobacter cloacae (100%, 9/9) and Klebsiella pneumoniae (100%, 7/7) isolates tested positive for ESBL production. Among Pseudomonas spp., 66.7% (2/3) of strains produced ESBLs, while the remaining strain (33.3%) tested negative. In contrast, E. coli isolates (0%, 0/3) did not exhibit ESBL production. AmpC production was detected in 9.1% (2/22) of isolates, limited to Enterobacter cloacae (22.2%, 2/9). No AmpC production was observed in Klebsiella pneumoniae (0%, 0/7), Pseudomonas spp. (0%, 0/3), or E. coli (0%, 0/3). No MBL or Carbapenemase activity was detected in any of the tested isolates (0%, 0/22). All Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas spp., and E. coli isolates tested negative for these resistance mechanisms. A summary of resistance mechanism prevalence across bacterial species is presented in Table 2.

Table 2. Prevalence of Resistance Mechanisms in Gram-Negative Bacteria Isolated from Diabetic Foot Ulcers.

Bacteria	Total Strains	ESBL Production (%)	AmpC Production (%)	Metallo Beta-Lactamase (%)	Carbapenemase (%)
Enterobacter cloacae	9	100.0 (9/9)	22.2 (2/9)	0.0 (0/9)	0.0 (0/9)
Klebsiella pneumoniae	7	100.0 (7/7)	0.0 (0/7)	0.0 (0/7)	0.0 (0/7)
Pseudomonas Spp.	3	66.7 (2/3)	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)
E. coli	3	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)	0.0 (0/3)
Total	22	81.8 (18/22)	9.1 (2/22)	0.0 (0/22)	0.0 (0/22)

ESBL = Extended-Spectrum Beta-Lactamase, MBL = Metallo Beta-Lactamase.

Biofilm Formation among Gram-Negative Bacteria

As shown in Figure 2, the average optical density (OD595nm) values indicate significant differences in biofilm formation across bacterial species. An ANOVA test confirmed these differences, yielding an F-value of 9.765 and a p-value of 0.000479, which is below the 0.05 threshold for statistical significance. Among the tested bacteria, Enterobacter cloacae and Klebsiella pneumoniae exhibited the highest biofilm formation, with similar OD595nm values. In contrast, E. coli displayed the lowest biofilm formation, as indicated by its lower OD595nm readings compared to the other bacteria. Pseudomonas spp. Demonstrated moderate biofilm formation, with OD595nm values higher than E. coli but lower than Enterobacter cloacae and Klebsiella pneumoniae. These results indicate a statistically significant variation in biofilm-forming capacity among the tested bacterial species, with Enterobacter cloacae and Klebsiella pneumoniae showing the highest levels of biofilm production under the studied conditions (Figure 2).

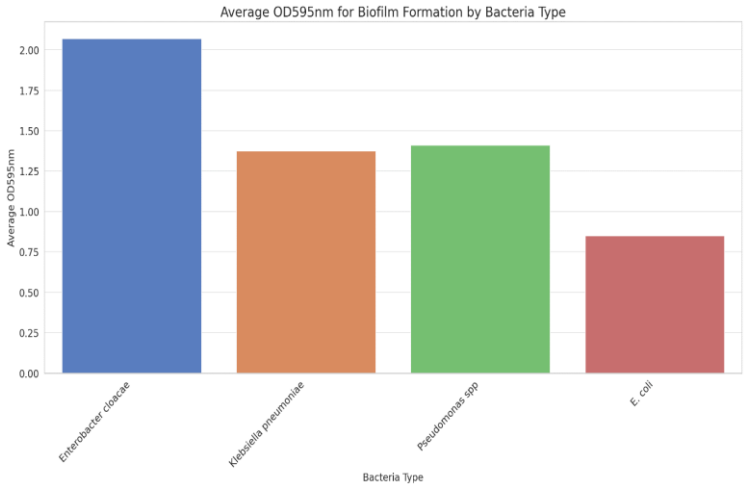

Figure 2. The average optical density (OD595nm) of biofilm formation by the tested bacteria

Plasmid-Associated Antibiotic Resistance Patterns

A comparative analysis of antibiotic resistance between plasmid-positive and plasmid-negative isolates was conducted to assess the impact of plasmid content on bacterial resistance profiles (Table 3). Statistical analysis using Welch's t-test revealed significant differences in mean inhibition zones for several antibiotics. As shown in Table 3, TE10, CIP5, LEV5, and FOX exhibited significantly higher resistance in plasmid-positive isolates compared to plasmid-negative isolates. For TE10, the mean inhibition zone decreased from 22.33 mm in plasmid-negative isolates to 16.47 mm in plasmid-positive isolates (p = 0.004). Similarly, CIP5

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resistance increased in plasmid-positive isolates, with a mean inhibition zone reduction from 31.00 mm to 24.00 mm (p = 0.002). The most pronounced difference was observed for LEV5, where plasmid-positive isolates had a mean inhibition zone of 11.80 mm compared to 30.50 mm in plasmid-negative isolates (p = 0.001). FOX resistance was also significantly higher in plasmid-positive strains, with a mean inhibition zone of 13.80 mm versus 19.00 mm in plasmid-negative strains (p = 0.045). In contrast, CN10 and AMC30 showed no significant correlation with plasmid content. CN10 inhibition zones remained similar between plasmid-positive (21.47 mm) and plasmid-negative (20.00 mm) isolates (p = 0.244). Likewise, AMC30 resistance was comparable across both groups, with plasmid-negative strains displaying complete resistance and plasmid-positive strains exhibiting an insignificant inhibition zone of 0.47 mm (p = 0.353). CAZ and CTX showed universal resistance, with both plasmid-positive and plasmid-negative isolates exhibiting 0 mm inhibition zones, indicating that resistance to these antibiotics is intrinsic and unrelated to plasmid content (Table 2). Species-specific trends were also observed. Enterobacter cloacae demonstrated increased resistance to TE10, LEV5, and CIP5 in plasmid-positive isolates compared to plasmid-negative isolates. Similarly, Klebsiella pneumoniae exhibited significant plasmid-associated resistance to FOX and TE10. In Pseudomonas spp., resistance to CAZ and CTX was universal, but CIP5 and FOX resistance was notably higher in plasmid-positive strains. E. coli also displayed increased resistance to TE10 and CIP5 in plasmid-positive isolates compared to plasmid-negative isolates.

Table 3. Comparison of Antibiotic Resistance Between Plasmid+ and Plasmid− Strains

Antibiotic	Mean Zone (Plasmid+), mm	Mean Zone (Plasmid−), mm	t-statistic	p-value	Significance (p < 0.05)	Trend
TE10	16.47	22.33	-3.45	0.004	Yes	Higher resistance in plasmid+
CN10	21.47	20.00	1.22	0.244	No	No significant trend
CIP5	24.00	31.00	-4.12	0.002	Yes	Higher resistance in plasmid+
CTI10	11.07	10.00	-0.75	0.455	No	No significant trend
LEV5	11.80	30.50	-5.32	0.001	Yes	Higher resistance in plasmid+
IMP	30.07	31.00	-0.45	0.652	No	No significant trend
CAZ	0.00	0.00	N/A	N/A	No	Universal resistance
CTX	0.00	0.00	N/A	N/A	No	Universal resistance
AMC30	0.47	0.00	0.95	0.353	No	Slight resistance in plasmid+
CRO	14.67	15.00	-0.23	0.821	No	No significant trend
FOX	13.80	19.00	-2.15	0.045	Yes	Higher resistance in plasmid+

Discussion

The management of diabetic foot ulcer (DFU) infections remains a significant clinical challenge, particularly due to the emergence of resistant Gram-negative bacteria. In our study, Enterobacter cloacae and Klebsiella pneumoniae were the most frequently isolated bacterial species, accounting for 47.6% and 35.7% of cases, respectively. This finding aligns with previous studies, which have reported these species as dominant DFU pathogens, notably in regions where antibiotic resistance is prevalent. Moreover, our findings contrast with previous reports, such as Rezazadeh, Hajian (27), who identified Staphylococcus aureus (34.5%) and Pseudomonas aeruginosa (30.4%) as the most prevalent bacteria in DFUs. In contrast, our study found Enterobacter cloacae (47.6%) and Klebsiella pneumoniae (35.7%) to be the dominant pathogens, with Pseudomonas spp. accounting for only 8.3% of isolates. These differences may reflect geographic variations in microbial etiology, differences in patient demographics, or institutional antibiotic prescribing patterns. Such disparities underscore the importance of local microbial surveillance to guide effective empirical antibiotic therapy for DFU management. Understanding these variations is critical for developing region-specific antimicrobial stewardship programs (28).

Our findings reveal alarming resistance patterns in DFU-associated Gram-negative bacteria, with E. coli, Enterobacter cloacae, and Klebsiella pneumoniae exhibiting 100% resistance to Ceftazidime (CAZ) and Amoxicillin-Clavulanic Acid (AMC30). This widespread resistance is consistent with findings from various

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studies, including those by Sadeghpour, Sharif (29), who reported high resistance to penicillin and ampicillin in UTI-associated Gram-negative bacteria, particularly E. coli and Klebsiella spp. However, their study found lower resistance rates to ciprofloxacin and gentamicin, whereas our findings indicate higher resistance to fluoroquinolones, particularly Levofloxacin (LEV5). These discrepancies suggest variability in antibiotic susceptibility based on infection site, emphasizing the importance of infection-specific resistance surveillance to guide empirical therapy (30). Alikhani, Banderas (31) found a high prevalence of antibiotic resistance in Pseudomonas aeruginosa, notably to cotrimoxazole (61.3%), and class 1 integrons in 57% of isolates, indicating a high potential for horizontal gene transfer and multidrug resistance (MDR). While our study did not assess genetic resistance determinants, the 100% resistance to CAZ and AMC30 in our isolates suggests possible plasmid- or integron-mediated resistance, consistent with Alikhani et al.'s findings. This highlights the risk of transferable resistance elements in hospital-acquired infections, further complicating DFU treatment strategies (30, 32).

Bababeekhou, Karshenasan (33) reported significant imipenem resistance (51.4%) and multidrug resistance (67.3%) in Pseudomonas aeruginosa, especially in metallo-beta-lactamase (MBL)-producing strains. Although MBL and carbapenemase activity were not detected in our study, increasing resistance to newer cephalosporins (ceftolozane, CTI10) and fluoroquinolones (LEV5) suggests that even last-resort antibiotics may be losing efficacy in diabetic foot ulcer (DFU) management, mirroring the trends observed by Babaeekhou et al. This underscores the urgent need for updated treatment guidelines incorporating local resistance surveillance and antibiotic stewardship to improve patient outcomes and curb resistance spread.

A high prevalence of Extended-Spectrum Beta-Lactamase (ESBL)-producing isolates was observed, particularly among Enterobacter cloacae and Klebsiella pneumoniae (100% positivity, Table 2). This aligns with the growing body of evidence indicating a global surge in ESBL-producing strains. Notably, the absence of ESBL production in E. coli in our study contrasts with findings from, suggesting possible strain-specific variations in ESBL expression (34, 35).

The restricted presence of AmpC β-lactamases to Enterobacter cloacae (22.2% positivity) indicates that ESBLs remain the dominant resistance mechanism in DFU infections. Encouragingly, no isolates exhibited Metallo Beta-Lactamase (MBL) or Carbapenemase activity, which is promising, as these resistance mechanisms are often associated with severe treatment failures. However, continued surveillance is necessary to monitor the potential emergence of carbapenem-resistant strains, as resistance trends may evolve.

Biofilm production is a major contributor to chronic wound infections, enabling bacteria to evade host immune responses, persist within the wound environment, and resist antibiotic penetration. Our study confirms that Enterobacter cloacae and Klebsiella pneumoniae exhibited the highest biofilm-forming capacity, consistent with previous findings (36, 37). The presence of strong biofilm producers in DFUs suggests that conventional antibiotic therapy alone may be insufficient, reinforcing the need for biofilm-targeting strategies, such as enzymatic biofilm disruptors, combination therapies, and antimicrobial dressings. Clinically, the presence of strong biofilm formers highlights the need for early intervention strategies, including debridement, prolonged antibiotic therapy, and adjunctive therapies to enhance biofilm disruption and wound healing (14, 38).

Plasmid-mediated resistance was particularly notable for TE10, CIP5, LEV5, and FOX, where plasmid-positive isolates exhibited significantly higher resistance (Table 3). This observation aligns with previous reports highlighting the critical role of plasmids in the dissemination of multidrug-resistant (MDR) genes through horizontal gene transfer (HGT) (39, 40). Specifically, Wang, Wang (40) demonstrated that mcr-1 encoding E. coli strains exhibited enhanced resistance due to plasmid-borne resistance determinants, reinforcing the link between plasmids and antimicrobial resistance. Similarly, Feng, Xu (39) confirmed that plasmid-mediated transmission of the mcr-1 gene is a dominant mechanism facilitating MDR spread in Gram-negative bacteria.

The ability of plasmids to disseminate resistance determinants within hospital environments poses a major infection control challenge, increasing the risk of nosocomial outbreaks of MDR pathogens (41). Talat, Khan (41) further illustrated how high-risk colistin-resistant Klebsiella pneumoniae strains acquired resistance due to plasmid-mediated blaNDM-5 genes, underscoring the role of hospital-acquired plasmid transmission in sustaining MDR infections.

However, our study found no significant correlation between plasmid content and resistance to CN10 (Gentamicin) and AMC30 (Amoxicillin-Clavulanic Acid), suggesting that other genetic mechanisms (e.g., chromosomal mutations or efflux pumps) may contribute to resistance in these cases. This aligns with the findings of Irusan, Akshay (42), who demonstrated that in Gram-negative clinical isolates, resistance to certain antibiotics, including aminoglycosides and β-lactams, was often mediated by chromosomal elements rather than plasmids.

Additionally, the universal resistance to CAZ (Ceftazidime) and CTX (Cefotaxime) in both plasmid-positive and plasmid-negative isolates suggests an intrinsic resistance mechanism unrelated to plasmid acquisition. This observation is consistent with Zhang, Yin (43), who identified that β-lactamase genes (such as blaNDM) play a dominant role in cephalosporin resistance, independent of plasmid-mediated factors. Their genomic characterization of Citrobacter freundii clinical strains confirmed that cephalosporin resistance was

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primarily driven by chromosomal β-lactamase genes rather than horizontally acquired plasmid genes.
Our findings reinforce the dual role of plasmids and chromosomal mutations in MDR. While plasmid-mediated resistance remains a dominant mechanism for key antibiotics (TE10, CIP5, LEV5, FOX), the presence of alternative resistance mechanisms (e.g., intrinsic β-lactamase production, efflux pumps, and chromosomal mutations) must also be considered when evaluating MDR profiles. Given the increasing complexity of resistance patterns, alternative therapeutic approaches and stringent infection control strategies will be critical to mitigating MDR pathogen outbreaks in hospital settings.
A key limitation of our study is that resistance characterization was solely based on the presence or absence of plasmids, as our facility lacked the necessary resources for detailed plasmid sequencing and functional analysis. This constraint prevents a comprehensive assessment of specific resistance genes harbored within plasmids and their exact contribution to antimicrobial resistance. Additionally, other genetic elements, such as integrons, transposons, chromosomal mutations, and efflux pumps, which may independently contribute to resistance, were not analyzed in depth. Future studies incorporating whole-genome sequencing (WGS) and transcriptomic analysis will be crucial in providing a more detailed understanding of the genetic determinants influencing multidrug resistance.

Conflict of interest. Nil

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