Section: Microbiology

Original Research Article

BACTERIOLOGICAL PROFILE, BIOFILM PRODUCTION AND ANTIMICROBIAL SUSCEPTIBILITY PATTERNS IN DIABETIC FOOT ULCERS

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Received *: 02/06/2025*
Received in revised form *: 16/07/2025*
Accepted *: 07/08/2025*

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DOI: 10.70034/ijmedph.2025.3.276

Source of Support: Nil,
Conflict of Interest: None declared

Int J Med Pub Health
2025; 15 (3); 1502-1507

ABSTRACT

Background: Diabetic foot ulcers (DFUs) are one of the most serious complications of diabetes mellitus, often associated with polymicrobial infections, multidrug resistance, and biofilm formation. Biofilm-producing bacteria contribute to poor wound healing and frequent treatment failures. Understanding local microbial patterns, resistance profiles, and biofilm production is critical for guiding appropriate therapy. The present study aimed to determine the bacterial profile, antibiotic susceptibility patterns, and biofilm-producing ability of isolates from DFUs in a tertiary care hospital.

Materials and Methods: A cross-sectional study was conducted over one year, including 139 diabetic patients with clinically diagnosed DFUs. Samples were collected aseptically, processed by standard microbiological methods, and identified using conventional techniques. Antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion method according to CLSI guidelines. Biofilm production was assessed qualitatively using the Congo Red Agar method. Data were analyzed using SPSS, and statistical significance was determined using Chi-square or Fisher's exact test.

Results: Bacterial growth was obtained in 72 (51.8%) samples, yielding 92 isolates. Monomicrobial infections were predominant (84.7%). The most common pathogens were Pseudomonas aeruginosa (18.5%), Klebsiella spp. (18.5%), Staphylococcus aureus (MSSA 17.4%, MRSA 14.1%), Escherichia coli (13%), and coagulase-negative staphylococci (14.1%). Biofilm production was significantly associated with P. aeruginosa (p=0.002) and E. coli (p=0.039). Biofilm-producing isolates showed higher resistance to ciprofloxacin, aminoglycosides, and amoxiclav, whereas amikacin and imipenem retained good activity against most Gram-negative isolates.

Conclusion: Biofilm formation is strongly associated with multidrug resistance in DFUs, particularly among P. aeruginosa and E. coli. Early biofilm detection and culture-directed therapy, along with exploration of novel antimicrobials including natural agents, are essential to improve clinical management.

Keywords: Diabetic foot ulcers, biofilm, multidrug resistance, Pseudomonas aeruginosa, Escherichia coli, antibiotic susceptibility.

INTRODUCTION

Diabetes mellitus is a chronic metabolic condition that causes hyperglycemia due to abnormalities in insulin production, insulin action, or both. Across the globe, it impacts millions of people, and its incidence is rising as a result of obesity, poor diet, and sedentary lifestyles. About 537 million persons

worldwide had diabetes in 2021, and by 2030, that figure is predicted to increase to 643 million, according to the International Diabetes Federation (IDF).[1]

Numerous acute and long-term consequences are linked to diabetes. Retinopathy, nephropathy, and neuropathy are examples of microvascular chronic consequences. Cardiovascular disease, cerebrovascular accidents, and peripheral artery disease are examples of macrovascular chronic complications. The emergence of diabetic foot ulcers (DFUs), which have a major negative impact on quality of life and, if left untreated, can result in lower extremity amputations, is one of the most damaging and expensive consequences.[2]

DFUs are full-thickness wounds below the ankle that typically occur in diabetic individuals due to immunological dysfunction, peripheral vascular disease, and neuropathy.[3] About 15–25% of people with diabetes have foot ulcers at some point in their lives.[4] Neuropathy causes a loss of protective feeling, and ischaemia and compromised immunological responses make the body less able to repair and more vulnerable to infections. Numerous bacteria frequently colonise or infect ulcers, which makes the wound environment and treatment results more difficult to manage.

Gram-positive cocci, gram-negative bacilli, and aerobic and anaerobic organisms make up the varied microbial flora of DFUs. Staphylococcus aureus, Streptococcus species, Enterococcus species, Escherichia coli, Klebsiella species, Proteus species, Pseudomonas aeruginosa, and anaerobes such as Bacteroides species are among the most often isolated pathogens.[5,6] Methicillin-resistant S. aureus (MRSA) and other strains of S. aureus are thought to be the most frequent cause of both acute and chronic infections.[7] Chronic ulcers are more likely to have polymicrobial infections, especially if they have significant tissue involvement or have been treated with antibiotics in the past.[7]

Antibiotic resistance among bacterial isolates from DFUs is a growing concern globally. Numerous interconnected variables contribute to antibiotic resistance in diabetic foot infections. The most common reason is the indiscriminate and empirical application of broad-spectrum antibiotics without first obtaining microbiological proof, which exposes bacteria under selection pressure. Subtherapeutic dosage and incomplete treatment regimens also aid in the adaptability and survival of resistant microorganisms.[8]

Biofilms are challenging communities of bacteria that stick to inert or alive surfaces, such as chronic wounds, by embedding themselves in an extracellular polymeric substance (EPS). Biofilm-forming bacteria are linked to treatment resistance, delayed wound healing, and persistent infection in DFUs.[9] Biofilms are thought to be present in 60–80% of chronic wounds, including diabetic ulcers.[10]

Understanding the regional microbialspectrum, resistance patterns, and predominance of species that produce biofilm is still lacking despite advancements in wound care. Finding these variables is essential to creating focused antimicrobial treatments and enhancing patient outcomes. The present study aims to analyze the bacterial profile and antimicrobial resistance patterns of isolates from diabetic foot ulcers, with special emphasis on the detection and characterization of biofilm-producing organisms.

MATERIALS AND METHODS

This cross-sectional observational study was conducted in the Department of Microbiology at a tertiary care teaching hospital over a period of one year. The study population included diabetic patients presenting with clinically diagnosed diabetic foot ulcers (DFUs). Ethical approval was obtained from the Institutional Ethics Committee and informed written consent was obtained from all participants prior to sample collection.

Patients aged 18 years and above with a known history of diabetes mellitus and presenting with foot ulcers showing clinical signs of infection were included in the study. Patients were excluded if they were non-diabetic, immunocompromised (e.g., on chemotherapy or immunosuppressive drugs).

Based on previous studies, the estimated prevalence (p) of biofilm-producing bacteria in diabetic foot ulcers was taken as 34% (0.34).[1] With a 95% confidence interval and a 5% margin of error, the sample size was 139.

Depending upon the ulcer, specimens were collected aseptically from the base of the wound after cleaning and debridement to avoid contamination. Deep tissue samples aspirates were preferred over superficial swabs and were transported immediately to the microbiology laboratory. Samples were inoculated onto Blood agar, MacConkey agar, and anaerobic media where necessary, and incubated aerobically at 37°C for 18 to 24 hours. Isolated organisms were identified by standard microbiological techniques. Antimicrobial susceptibility testing (AST) was performed on all bacterial isolates using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar, following the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Biofilm production was detected by using the Congo Red Agar (CRA) method, a simple qualitative screening technique. Congo Red Agar was prepared using brain heart infusion (BHI) broth (37 g/L), sucrose (50 g/L), agar (10 g/L), and Congo red dye (0.8 g/L). The medium was sterilized, poured into sterile Petri plates, and allowed to solidify.

Bacterial isolates were streaked onto the CRA plates and incubated aerobically at 37°C for 24 to 48 hours. After incubation, colonies were observed. Black colonies with a dry, crystalline consistency

were considered strong biofilm producers, dark red to black colonies without a dry appearance were considered moderate/weak producers, and red, smooth colonies were interpreted as non-biofilm producers. This method provides a visual indication of slime production associated with biofilm-forming capacity.[11]

All data were compiled and entered into Microsoft Excel and analyzed using SPSS software. Descriptive statistics were used to summarize demographic data, microbial profiles, resistance patterns, and the prevalence of biofilm production. Associations between bacterial isolates, antibiotic resistance, and biofilm formation were analyzed using Chi-square or Fisher's exact test, with a p-value <0.05 considered statistically significant

RESULTS

A total of 139 patients with diabetic foot ulcers were included in the study. In the present study 72 samples (51.80%) yielded the growth. Out of 72 samples, 92 bacterial isolates were observed. Monomicrobial growth (84.72%) was observed in majority of samples followed by polymicrobial growth 15.28%). Male predominance (71.2%) was observed. Biofilm production was significantly more common among male patients (p = 0.0006). Analyzing the age distribution, the majority of patients were between 50–80 years, with no significant association found between age and biofilm production. Regarding the duration of diabetes, 40 patients had diabetes for less than five years and 99 for more than five years, but the difference in biofilm production between these groups was not statistically significant (p = 1.000).

In our study, the duration of the ulcer showed a strong correlation with biofilm production. Patients with ulcers present for more than three months exhibited significantly higher biofilm formation compared to those with a shorter ulcer duration (p = 0.0001). The site of the ulcer did not show a significant relationship with biofilm production (p = 0.101), nor did the type of anti-diabetic treatment, with no substantial difference between those receiving oral hypoglycemics and those on insulin therapy (p = 0.439). Table.1.

Table 1: Correlation of patient characteristics with bacterial isolates and biofilm formation

VARIABLE		NUMBER OF SAMPLES	FREQUENCY OF ISOLATES	BIOFILM PRODUCERS	P VALUE
Gender	Male	99	71	32	0.0006
	Female	40	21	19	
Age	38-50	12	1	1	-
	>50-65	45	20	20	
	>65-80	57	26	26	
	>80	25	4	4	
Duration of Diabetes	<5 Years	40	22	12	1.0000
	≥5 Years	99	70	39	
Duration of ulcer	≤ 1 Month	32	25	9	0.0001
	>1-3 Months	55	31	11	
	>Months	52	36	31	
Site of ulcer	Fore foot	71	51	25	0.1010
	Mid	22	18	14	
	Hand	46	23	12	
Type of Anti diabetes	Oral	43	25	16	0.4390
	Insulin	96	67	35	

The most commonly isolated organisms from diabetic foot ulcers were *Pseudomonas aeruginosa*, *Klebsiella* spp., *Staphylococcus aureus* (both MSSA and MRSA), *Escherichia coli*, and coagulase-negative staphylococci (CONS). Among these, *P. aeruginosa* and *E. coli* demonstrated statistically significant associations with biofilm production (p = 0.002 and p = 0.039, respectively). Other organisms such as MSSA, MRSA, *Klebsiella* spp., and CONS also showed biofilm production but without statistical significance. Table.2

Table 2: Bacterial isolates and their biofilm-forming ability

Bacteria	Isolates (n=92)	Biofilm Producers (n=53)	P-Value
Pseudomonas	17(18.48%)	15(28.30%)	**0.002**
E. coli	12(13.04%)	10(18.87%)	**0.039**
CONS	13(14.13%)	3(5.67%)	0.092
Staph. aureus (MSSA)	16(17.39%)	5(9.43%)	0.210
Staph. aureus (MRSA)	13(14.13%)	9(16.98%)	0.267
Klebsiella sp.	17(18.48%)	11(20.75%0	0.332
Proteus	2(2.17%)	0(0%	0.5
NFGNB	2(2.17%)	0(0%)	0.5

MSSA: Methicillin Sensitive Staphylococcus aureus; MRSA: Methicillin Resistance Staphylococcus aureus, NFGNB: Non fermenting gram negative bacilli.

Antibiotic susceptibility testing revealed a distinct pattern of increased resistance among biofilm-producing isolates. In *P. aeruginosa,* resistance was highest to ciprofloxacin and cefepime, with sensitivities of only 46.7% and 46.7%, respectively. *E. coli* biofilm producers also showed moderate resistance to several antibiotics, while remaining partially sensitive to amoxiclav and gentamicin. CONS were highly sensitive to vancomycin but resistant to most other agents. MSSA and MRSA biofilm producers showed variable sensitivity, with MRSA showing low susceptibility to ciprofloxacin and cephalosporins, though two-thirds remained sensitive to vancomycin. *Klebsiella* species among biofilm producers were highly sensitive to amikacin and imipenem (90.9%). Table.3

Table 3: Susceptability pattern of biofilm producing organisms

S.No	ORGANISM	CIP	AK	GEN	AMC	IMP	VA	CTR	CAZ	CFM
1	P.aeruginosa (n=15)	7 (46.67)	10 (66.67)	9 (60)	10 (66.67)	11(73.33)	NT	10 (66.67)	8 (53.33)	7 (46.67)
2	E.coli (n=10)	3 (30)	5 (50)	5 (50)	7 (70)	4 (40)	NT	5 (50)	3 (30)	4 (40)
3	CONS (n=3)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	NT	3 (100.0)	1 (33.3)	3 (100.0)	1 (33.3)
4	NSSA (n=5)	1 (20)	5 (100)	4 (80)	2 (40)	NT	5 (100)	1 (20)	1 (20)	1 (20)
5	MRSA (n=9)	1 (11.11)	2 (22.22)	1 (11.11)	0 (0.00)	NT	6 (66.67	3 (33.3)	1 (11.11)	1 (11.11)
6	Klbseilla species (n=11)	0 (0.00)	10 (90.91)	5 (45.45)	7 (63.64)	10 (90.91)	NT	5(45.45)	4 (36.36)	4 (36.36)
7	Proteus (n=0)	-	-	-	-	-	-	-	-	-
8	NFGNB (n=0)	-	-	-	-	-	-	-	-	-

CIP: Ciprofloxacin; AK: Amikacin; GEN: Gentamicin; AMC: Amoxycillin clauvulinic acid; IMP: Imipenam; VA: Vancomycin, CTR: Ceftriaxone; CAZ: Ceftazidime; CFM: Cefipime

In comparison, non-biofilm-producing organisms demonstrated higher susceptibility across all antibiotics tested. Both *P. aeruginosa* and *E. coli* non-biofilm producers were fully sensitive to all antibiotics tested. CONS and MSSA non-producers displayed high rates of sensitivity, especially to vancomycin and aminoglycosides. MRSA non-biofilm producers also retained partial sensitivity to amikacin and vancomycin, while *Klebsiella* species non-producers showed full sensitivity to imipenem and amikacin. *Proteus* spp. and non-fermenting gram-negative bacilli (NFGNB) were sensitive to all antibiotics in both biofilm and non-biofilm groups. Table.4

Table 4: Susceptability pattern of biofilm non producing organisms

S.No	ORGANISM	CIP	AK	GEN	AMC	IMP	VA	CTR	CAZ	CFM
1	P.aeruginosa (n=2)	1 (50.0)	2 (100.0)	1 (50)	2 (100)	2 (100)	NT	1 (50)	2 (100)	2 (100)
2	E.coli (n=2)	0 (0.0)	2 (100.0)	2 (100)	2 (100)	2 (100)	NT	2 (100)	1 (50)	1 (50)
3	CONS (n=10)	7 (70.0)	10 (100.0)	7 (70)	7 (70)	NT	10 (100)	5 (50)	6 (60)	7 (70)
4	NSSA (n=11)	11 (100.0)	11 (100.0)	11 (100)	10 (90.9)	NT	11 (100)	8 (72.7)	8 (72.7)	5 (45.5)
5	MRSA (n=4)	0 (0.0)	2 (50.0)	1 (25)	2 (50.0)	NT	3 (75)	0	0	0
6	Klbseilla species (n=6)	6 (100.0)	5 (83.3)	5 (83.3)	6 (100.0)	6 (100)	NT	0	3 (50)	2 (33.3)
7	Proteus (n=2)	2 (100.0)	2 (100.0)	2 (100)	2 (100.0)	2 (100)	NT	2 (100)	2 (100)	2 (100)
8	NFGNB (n=2)	0 (0.0)	2 (100.0)	1 (50)	1 (50.0)	2 (100)	NT	0	2 (100)	1 (50)

Biofilm-producing isolates exhibited significantly reduced susceptibility to ciprofloxacin, amikacin, gentamicin, and amoxiclav ($p \leq 0.01$), indicating a strong association between biofilm formation and multidrug resistance. In contrast, no significant difference in sensitivity was observed for imipenem, vancomycin, ceftriaxone, ceftazidime, and cefixime ($p > 0.05$), suggesting that biofilm production does not uniformly affect all antibiotic classes. Table. 5

Table 5: Antibiotic Susceptibility Comparison (Biofilm vs. Non-Biofilm)

Antibiotic	Mean Sensitivity (%) - Biofilm	Mean Sensitivity (%) - Non-Biofilm	Chi-Square	p-value
Cip	22.64	71.43	18.64	0.00
Ak	62.26	91.43	7.84	0.01
Gen	45.28	77.14	7.52	0.01
Amc	49.06	82.86	8.88	0.00
Imp	69.44	100.00	2.51	0.11
Va	82.35	96.00	0.89	0.35
Ctr	47.17	45.71	0.00	1.00
Caz	37.74	57.14	2.47	0.12
Cfm	33.96	48.57	1.32	0.25

DISCUSSION

In the present study, bacterial growth was observed in 72 out of 139 samples, yielding a culture positivity rate of 51.8%. The isolation rate aligns with earlier studies that report variability depending on the sample type and prior antibiotic exposure. Factors such as inadequate sampling depth or the use of empirical antibiotics before sample collection may account for culture-negative results in nearly half of the cases.[5,12] Among the positive cultures, monomicrobial infections were predominant (84.72%), whereas polymicrobial growth was noted in 15.28%, supporting existing literature which suggests that chronic ulcers may harbor diverse microbial communities, though often dominated by a single pathogen.[8]

In the present study, no significant association of biofilm production was observed in relation to age and duration of diabetes. This finding is in line with previous studies.[10] On the other hand, biofilm production was more prevalent in ulcers that lasted longer than three months, and the length of the ulcer was significantly correlated with biofilm development ($p = 0.0001$). Since bacteria in mature wounds have plenty of time to create organised communities embedded in extracellular polymeric substances that are resistant to medicines and immune clearance, this lends strength to the theory that chronicity of infection encourages the formation of biofilms.[13,14]

In the present study, Pseudomonas aeruginosa (18.48%), Klebsiella spp. (18.48%), Staphylococcus aureus (MSSA 17.39%, MRSA 14.13%), Escherichia coli (13.04%), and coagulase-negative staphylococci (CONS 14.13%) were the predominant isolates. Among these, P. aeruginosa ($p=0.002$) and E. coli ($p=0.039$) showed a statistically significant association with biofilm production, whereas other organisms such as Klebsiella spp., MSSA, and MRSA, though biofilm producers, were not statistically significant. This finding is consistent with previous reports indicating that Gram-negative bacilli, particularly P. aeruginosa and E. coli, are frequent biofilm producers in diabetic foot infections (DFIs).[9,10]

However, some studies have reported different predominant pathogens. A global meta-analysis found Staphylococcus aureus to be the leading isolate in DFIs, followed by P. aeruginosa and Enterobacterales.[15] In contrast, Indian studies generally show Gram-negative predominance, with E. coli, P. aeruginosa, and Klebsiella spp as the major pathogens.[16] An Indian meta-review of 54 studies also reported high frequencies of S. aureus (76%) and Enterococcus spp. (31%), although Gram-negative organisms collectively accounted for the majority of isolates.[17]

In our study, biofilm-producing isolates showed markedly higher resistance compared to non-biofilm producers. Pseudomonas aeruginosa biofilm producers were less susceptible to ciprofloxacin (46.7%) and cefepime (46.7%), while moderately sensitive to amikacin (66.7%) and imipenem (73.3%). E. coli biofilm producers exhibited resistance to ciprofloxacin (30%) and cefepime (40%) but retained partial sensitivity to amoxiclav (70%) and gentamicin (50%). Klebsiella spp. biofilm producers remained highly susceptible to amikacin (90.9%) and imipenem (90.9%). MRSA biofilm producers showed poor susceptibility to fluoroquinolones and cephalosporins but were largely sensitive to vancomycin (66.7%). Non-biofilm-producing isolates of all species demonstrated higher sensitivity across most antibiotics.

These findings are consistent with previous studies which established that biofilm formation in diabetic foot infections (DFIs) is a major contributor to multidrug resistance (MDR) Biofilms impede antibiotic penetration, facilitate horizontal gene transfer, and create a protected niche where bacteria persist despite antimicrobial exposure.[8,10]

Similarly, .Shanmugam et al. reported that biofilm-producing strains of P. aeruginosa, E. coli, and Klebsiella were predominantly MDR and significantly less responsive to conventional antibiotics.[8] The high susceptibility of Klebsiella biofilm producers to amikacin and imipenem in our study aligns with other Indian reports where carbapenems and aminoglycosides remain effective options against biofilm-associated Gram-negative pathogens.[13] However, the reduced activity of ciprofloxacin and third-generation cephalosporins against P. aeruginosa and E. coli biofilm producers is concerning, as these drugs are commonly used empirically in DFIs.

Vancomycin retained its efficacy against MRSA and coagulase-negative staphylococci (CONS), in agreement with earlier studies underscoring its role as a key agent for Gram-positive coverage in biofilm-associated DFIs.[18] However, the emergence of MDR biofilm-producing Gram-negatives highlights the urgent need for biofilm-targeted strategies such as combination therapy, debridement, and novel antibiofilm agents.[14]

This study has certain limitations. Biofilm detection was performed using the Congo Red Agar (CRA) method, which, although simple and cost-effective, is qualitative and less sensitive compared to quantitative methods such as the microtiter plate assay. Additionally, anaerobic culture methods were not employed, which may have led to underestimation of the complete microbial profile of diabetic foot ulcers, particularly in chronic or deep-seated infections. Furthermore, the study was conducted in a single center with a limited sample size, which may restrict the generalizability of the findings.

CONCLUSION

The present study explored, Pseudomonas aeruginosa and E. coli as the predominant biofilm-producing pathogens in diabetic foot ulcers, significantly associated with antimicrobial resistance. While Klebsiella spp. remained susceptible to amikacin and imipenem, MRSA showed sensitivity to vancomycin, underscoring the value of culture-guided therapy. These findings highlight the importance of early biofilm detection and the potential role of new antimicrobials derived from natural substances as adjunctive therapies to overcome biofilm-related resistance and improve healing outcomes.

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