Content available at: https://www.ipinnovative.com/open-access-journals

Indian Journal of Microbiology Research

Journal homepage: www.ijmronline.org

Original Research Article

Correlation of antimicrobial resistance, biofilm production of bacteria infecting grade 2 and 3 diabetic foot ulcers

MasoodAhmed Tahashildar[1,2], Sheetal Harakuni[2]*, Shrishail Metgud[3], Basavaraj M Kajagar[3], Manoj Togale[3]

[1]Dr. Prabhakar Kore Basic Science Research Center, KAHER, Belagavi, Karnataka, India
[2]Dept. of Microbiology, JN Medical College, KLE Academy of Higher Education and Research, Belagavi, Karnataka, India
[3]Dept. of Surgery, JN Medical College, KLE Academy of Higher Education and Research, Belagavi, Karnataka, India

Abstract

Background: Diabetes is a notorious metabolic disorder characterized by long-term complications such as chronic diabetic foot ulcers (DFUs). DFUs pathogenesis is a product of complex factors lead by long-term diabetic mellitus. The ulcers usually get infected and make treatment complicated.
Objective: The present study aimed to explore the connection between patterns of antimicrobial sensitivity, biofilms formation, as well as severity in chronic foot ulcers.
Materials and Methods: A total of 86 wound/pus sample collected from patients presenting with grade-2 and grade-3 chronic DFUs, were processed for microbial identification and antibiotic sensitivity (CLSI – 2022 guidelines). All isolated bacteria were subjected to biofilm detection test by microtiter plate method. Spearman's correlation coefficient calculated for each pair of variables to investigate the relationships between biofilm formation, resistance score and diabetic foot ulcer grades.
Results: Out of 110 isolates, biofilm detection assay found five strong biofilm formers, 9 medium biofilm formers, and 22 isolates found to be low biofilm formers. The Spearman's correlation analysis between antibiotic resistance and biofilm formation was found to be positive, there was a weak relationship between ulcer grades and biofilm formation. However, there was no significant relationship between resistant score and ulcer grades.
Conclusion: Biofilm forming ability needs to be screened among the isolates from a chronic diabetic wound as they facilitate antibiotic resistance. Thus, there is a need for reconsideration of treatment modalities, implementation of effective infection control practices, novel therapies and patient education.
Keywords: Diabetic foot, Foot ulcer, Biofilms, Drug resistance.

Received: 12-08-2025; **Accepted:** 07-09-2025; **Available Online**: 20-09-2025

1. Introduction

Diabetic foot ulcers are prevalent long-term sequelae of diabetes. About 19-34% of diabetics suffer from chronic foot ulcers. Managing these ulcers can lead to higher healthcare costs, longer hospital stays, and an increased risk of limb amputation, adding to the morbidity and consequences on healthcare system.[1-4]

Diabetic foot ulcers (DFUs) pathogenesis is a complex combination of factors including metabolic dysfunction, immunopathy, diabetic neuropathy, and vascular impairment. DFUs characterized by insufficient glucose metabolism, decreased growth factor activity, elevated pro-inflammatory cytokines, impaired angiogenesis and dysregulation in matrix metalloproteinases synthesis and degradation, all of which are exacerbated by microbial infections.[5-7]

There is a consensus that among chronic wounds biofilm forming pathogens facilitate secondary infections and the wounds are refractory to the conventional treatment.[8] The emergence of multidrug-resistant (MDR) pathogens has made treating chronic diabetic foot ulcers more difficult.[9-11] While previous research has explored biofilm formation and

*Corresponding author: Sheetal Harakuni
Email: sheetalharakuni@gmail.com

https://doi.org/10.18231/j.ijmr.33816.1758360749

antimicrobial resistance in diabetic foot ulcers (DFUs), there is limited understanding of how these factors correlate with the severity of chronic DFUs, particularly in grades 2 and 3. This study provides a comprehensive analysis of the correlation between biofilm formation, antimicrobial resistance, and DFU grades 2 and 3. By examining these relationships, the research offers novel insights into the complex interplay between bacterial characteristics and ulcer severity in chronic DFUs.

2. Material and Methods

The study employed a cross-sectional design and was conducted over a one-year period at a tertiary care hospital and research facility in North Karnataka, India. The study protocol was reviewed and approved by the Institutional Review Board. The individuals presenting Type 2 diabetes mellitus diagnosed with chronic foot ulcers of grade 2 and 3 were included in present study. Ulcers classified using the *Wagner-meggitts* classification[12] by expert clinicians.

2.1. Sample collection

The study population comprised 86 individuals presenting with type 2 diabetes mellitus with chronic diabetic foot ulcers were included, these patients were selected based on specific inclusion criteria, which likely considered factors such as the duration of diabetes, severity of the foot ulcer, and the absence of recent antibiotic treatment. The process of sample collection from chronic wounds in diabetic foot ulcers involved aspirating pus and swabbing using the Levine technique. Pus aspiration directly extracts fluid from the chronic wound, while in the Levine technique entails applying by rotating a swab with firm pressure over a 1 cm^2 wound area to collect tissue fluid. These samples were then carefully transported to the microbiology laboratory in sterile test tubes, ensuring that processing occurs within 6 hours to maintain the integrity of the microbial populations present. Along with the pus samples, comprehensive demographic information was collected from each patient. The data collected included age, gender, duration of diabetes, glycemic control status and other relevant medical history, offering a comprehensive view of the patient population.

2.2. Microbial detection, identification and characterization

Samples were inoculated onto blood agar and MacConkey agar and incubated at 37°C for 24 h. Predominant isolates were selected in cases of polymicrobial growth, if two or more organisms were predominant, known pathogens were identified and maintained in stock. Isolates were characterized and identified using standard biochemical methods[13] and antimicrobial susceptibility was determined by Kirby–Bauer disc diffusion assay in line with CLSI M100 (2022) guidelines.[14] To assess how resistant each isolate was, we calculated a resistance index. This was done by dividing the number of antibiotics the isolate resisted by the total number of antibiotics it was tested against. The index provided a simple measure of the overall resistance level of each isolate.[15]

2.3. Biofilm formation assay

The biofilm detection was carried out using the Tissue Culture Plate (TCP) method, as described by Stepanovic *et al.*,[16] This method involves culturing bacteria in 100 µl Brain-Heart Infusion (BHI) broth supplemented with glucose 1%, followed by incubation at 37°C for 18-24 hours in 96-well plates, each isolate was inoculated in triplets. Following incubation, wells were washed to remove free floating bacterial cells, and the adherent biofilms stained with crystal violet. The optical density (OD) of the stained biofilm was determined spectrophotometrically to quantify biofilm formation.

The categorization of biofilm forming ability was evaluated by comparison of isolates OD to that of the negative control (ODc). This system classifies bacterial isolates were divided into four groups: those that do not develop biofilm ($OD \leq ODc$), those that do ($ODc < OD \leq 2\times Odc$), those that do so moderately ($2\times ODc < OD \leq 4\times Odc$), and those that do so strongly ($OD > 4\times Odc$).[16] This standardized approach allows for consistent evaluation and comparison of biofilm formation across different bacterial species

2.4. Statistical analysis

Statistical tests were done using SPSS 30 software. The percentages of resistance index and biofilm grades were calculated. Spearman's correlation coefficient was calculated for each pair of variables to investigate the relationships between biofilm formation, antimicrobial resistance, and diabetic foot ulcer grades.

3. Results

Of the 86 chronic DFUs patients, 110 bacterial pathogens were isolated. The predominant pathogens identified as *S aureus, P aeruginosa, K pneumoniae, and P vulgaris* **(Table 1).** All of them were further tested for biofilm formation assay, and out of 110 isolates, 46 were found to be biofilm formers which were further categorized as mentioned in **Table 2.**

3.1. Antimicrobial resistance patterns

We analyzed the antimicrobial resistance patterns of 110 isolates obtained from patients with chronic diabetic foot ulcers to correlate them with their biofilm-forming ability. The resistance index was calculated for each of the bacterial isolates. We found that the resistance score of the non-biofilm formers was higher than that of biofilm formers. Which explains the biofilm formers have high resistance to antibiotics as compared to the non-biofilm forming isolates **(Table 3).**

Table 1: Different bacterial species isolated from grade-2 and grade-3

Bacterial Species	**Grade 2**	**Grade 3**
S. aureus	19	17
S. epidermidis	1	2
P. vulgaris	9	5
P. mirabilis	3	2
P. aeruginosa	10	5
K. pneumoniae	13	8
E. coli	8	6
A. baumannii	1	0
E. aerogenes	0	1
Total	64	46
	110	

3.2. Biofilm formation and antimicrobial resistance score

In the biofilm analysis 46(41.81%) isolates were biofilm-forming according to the microtiter plate method, on scoring for biofilm formation five were Strong (10.86%), 19 Medium (41.30%), and 22(47.82%) were low biofilm formers. Further, Resistance score of each isolate correlated with biofilm-forming ability. A statistical correlation was observed between antimicrobial sensitivity pattern and biofilm formation ($p < 0.001$). (**Figure 1**)

3.3. Grade of diabetic foot ulcer, biofilm formation, and antimicrobial resistance

The ulcers grading for this biofilm forming bacterial isolates categorized based on the *Wagner-Meggitts* classification system and it was found that 27 patients presented with grade-2 and 19 patients with grade-3.

1. Biofilm formation and diabetic foot ulcer grades: Spearman's rank correlation coefficient used to determine the association between biofilm formation and DFUs grades. No statistically significant correlation was observed between ulcer grade and biofilm formation ($p = 0.418$) (**Figure 1**).

2. Antimicrobial resistance and diabetic foot ulcer grades: Spearman's rank correlation coefficient was used to understand the relation between antibiotic resistance and DFU grades. There was a weak negative correlation between the resistant score and ulcer grades. Therefore, it was considered to be not significant ($p = 0.519$). This finding suggests that variations in the resistant scores are not associated with variation in the ulcer grades (**Figure 2**).

Table 2: Different categories of Biofilm forming pattern among the isolated bacteria

Organisms	*S. aureus*	*S epidermidis*	*P. vulgaris*	*P. mirabilis*	*P. aeroginosa*	*K pnenomiae*	*E coli*	*E aerogenes*	**Total**
Category									
Low	6	1	4	2	2	4	2	1	22
Medium	7		2		3	4	3		19
Strong	2				1	1	1		5
Total	15	1	6	2	6	9	6	1	46

Table 3: Antibiotic resistance index for biofilm forming bacteria and non-biofilm forming bacteria

Bacterial isolate	**Resistance index (Cumulative)**		**p-value (Biofilm vs Non-biofilm)**
	Biofilm forming	**Non biofilm forming**	
S. aureus	1.33	2.7	0.056
P. aeruginosa	1.25	4	
S. epidermidis	5	3.7	
P. mirabilis	3	4	
P. vulgaris	3.6	5	
K. pnenomiae	4.5	5	
E. coli	1.6	3	
E. aerogenes	4	-	
A. baumannii	-	5	
Overall (Mean ± SD)	3.04 ± 1.47	4.05 ± 0.75	

Statistical analysis was done using Mann–Whitney U test. The overall statistical significance $p = 0.056$. Values with $p < 0.05$ were considered significant.

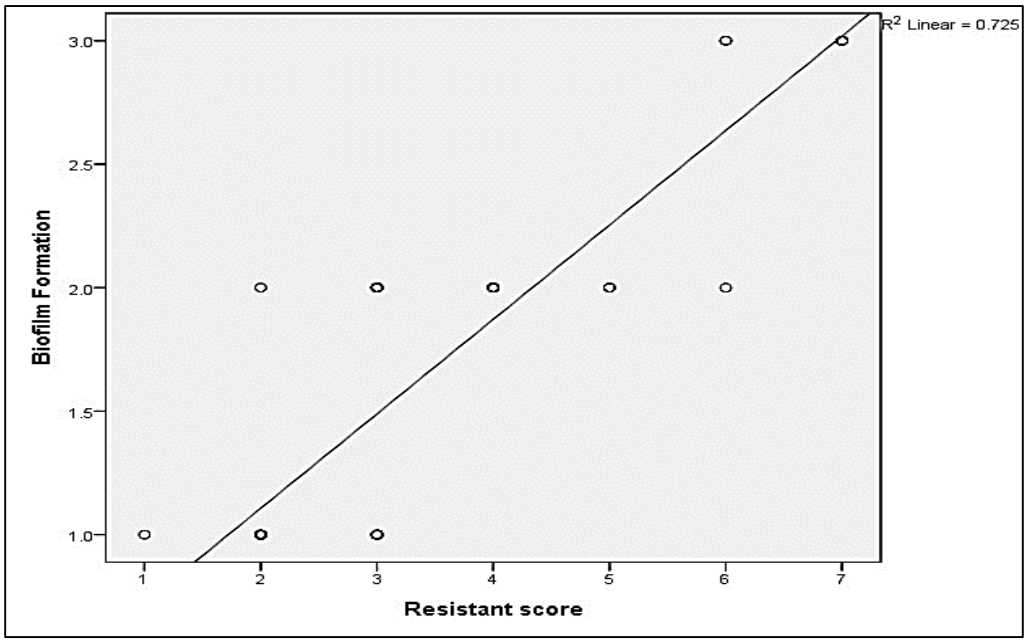

Figure 1: Scatter plot of biofilm formation vs. antimicrobial resistance score

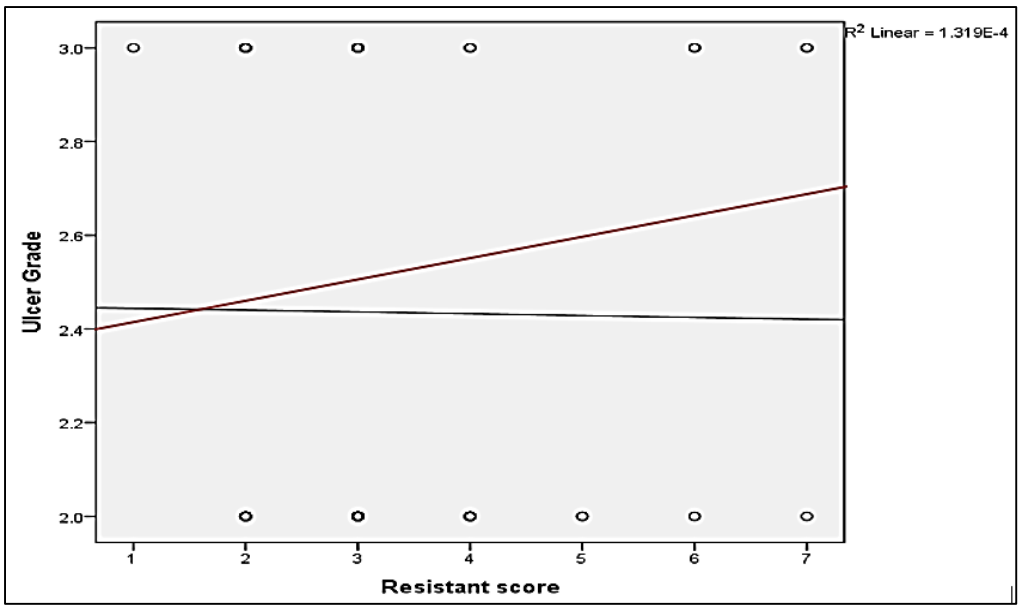

Figure 2: Scatter plot of ulcer grades vs. biofilm formation vs. resistance score. (*Red line indicates biofilm formation score against ulcer grades)

4. Discussion

The relationship between the different grades of diabetic foot ulcers, the biofilm forming strength of organisms and antibiotic resistance are the important findings of the present study. The obtained data exhibits a wide range of antibiotic resistance profiles and biofilm-forming abilities among distinct pathogens isolated from chronic DFUs.

The biofilm-forming abilities of different isolates vary. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli* are predominantly associated with biofilm formation. (**Table 1** and **Table 2**). These biofilms make treatment more difficult as they shield the bacteria against the host immune system and antibiotics.[17,18]

Our data indicates that antimicrobial resistance is highly prevalent among these isolated bacteria against several commonly used antibiotics, with an overall high resistance ratio in biofilm formers ranging from 1.3 to 4 compared to non-biofilm formers.(**Table 3**) The Present study outcome found to be consistent with the growing evidence of diabetic foot infections that are associated with higher antibiotic resistance due to the presence of biofilm-forming bacterias.[19,20]

The present findings reveal a clear link between higher ulcer severity, increased antimicrobial resistance, and biofilm formation. This implies that as ulcers progress, they are more likely to harbor bacteria that are resistant to the antibiotics and can form protective biofilms. Biofilm producing bacteria poses a significant challenge in antibiotic treatment, and their capacity to form biofilms contributes to ulcer severity and prolonged sustained presence of foot ulcers among susceptible population.[21] For advanced ulcers, more aggressive alternative treatment approaches may be required to effectively combat these difficult biofilms forming bacterial populations.[22,23]

This study highlights the resistance trends observed in biofilm-forming organisms and to improve our understanding of antibiotic resistance mechanisms over a period of time in biofilm-forming infections. A follow-up study with fresh wound samples collected at multiple time points may further reveal evolving antimicrobial resistance mechanisms and different microbial profiles.

5. Conclusion

In this study, bacteria from chronic DFUs were found to be both highly resistant to antibiotics and capable of forming biofilms. These results point to a complex link between biofilm formation and antibiotic resistance, underlining the need to take both factors into account when evaluating infections and planning treatment strategies.

6. Source of Funding

None.

7. Conflict of Interest

The authors report there are no competing interests to declare.

8. Ethical Approval

The study protocol was reviewed and approved by the Institutional Review Board of KLE Academy of Higher Education and Research with ref. no. KAHER/EC/23-24/41.

References

1. Armstrong DG, Boulton AJM, Bus SA. Diabetic foot ulcers and their recurrence. *N Engl J Med*. 2017;376(24):2367–75.
2. Long I. Insight of the pathophysiology of diabetic foot ulcer. In: Raghav A, editor. Diabetic Foot - Recent Advances [Internet]. IntechOpen; 2023 [cited 2024 Aug 13]. Available from: https://www.intechopen.com/chapters/84511
3. Kim J. The pathophysiology of diabetic foot: a narrative review. *J Yeungnam Med Sci*. 2023;40(4):328–34.
4. Peng Z, Lavigne JP. Pathogenesis of diabetic foot ulcers. *Front Pharmacol*. 2023;14:1218201.
5. Eckert AJ, Zimny S, Altmeier M, Dugic A, Gillessen A, Bozkurt L, et al. Factors associated with diabetic foot ulcers and lower limb amputations in type 1 and type 2 diabetes supported by real-world data from the German/Austrian DPV registry. *J Diabetes*. 2024;16(2):e13531.
6. Nawaz AD, Zaidi A, Elsayed B, Jemmieh K, Eledrisi M. Perspective chapter: epidemiology and risk factors of diabetic foot ulcer. In: Chowdhury EH, Zughaier SM, Hasan A, Alfkey R, editors. Diabetic foot ulcers - Pathogenesis, innovative treatments and AI Applications [Internet]. IntechOpen; 2024 [cited 2024 Aug 13]. Available from: https://www.intechopen.com/chapters/1166144
7. Hu L, Liu W, Yin L, Yi X, Zou Y, Sheng X. Analysis of factors influencing the recurrence of diabetic foot ulcers. *Skin Res Technol*. 2024;30(7):e13826.
8. Schultz G, Bjarnsholt T, James GA, Leaper DJ, McBain AJ, Malone M, et al. Consensus guidelines for the identification and treatment of biofilms in chronic nonhealing wounds. *Wound Rep Reg*. 2017;25(5):744–57.
9. Biswas SK, Roy SR, Paul AR. Bacterial isolates from diabetic foot ulcer with antibiotic sensitivity pattern. *Community Based Med J*. 2024;13(1):89–94.
10. Baral P, Afnan N, Ahmad Zahra M, Akter B, Rabia Prapti S, Muazzam Hossan M, et al. Bacteriological analysis and antibiotic resistance in patients with diabetic foot ulcers in Dhaka. *PLoS One*. 2024;19(5):e0301767.
11. Zafer MM, Mohamed GA, Ibrahim SRM, Ghosh S, Bornman C, Elfaky MA. Biofilm-mediated infections by multidrug-resistant microbes: a comprehensive exploration and forward perspectives. *Arch Microbiol*. 2024;206(3):101.
12. Parija SC. Textbook of Practical Microbiology. 2nd ed. Delhi: Tarun Ahuja, Ahuja Publishing House; 2018. p. 308.
13. Wang X, Yuan CX, Xu B, Yu Z. Diabetic foot ulcers: classification, risk factors and management. *World J Diabetes*. 2022;13(12):1049.
14. Wayne PK. Performance Standards for Antimicrobial Susceptibility Testing. Clinical and Laboratory Standards Institute; 2022.
15. Mir R, Salari S, Najimi M, Rashki A. Determination of frequency, multiple antibiotic resistance index and resistotype of *Salmonella* spp. in chicken meat collected from southeast of Iran. *Vet Med Sci*. 2022;8(1):229–36.
16. Stepanović S, Vuković D, Hola V, Bonaventura GD, Djukić S, Ćirković I, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS*. 2007;115(8):891–9.
17. Malik A, Mohammad Z, Ahmad J. The diabetic foot infections: biofilms and antimicrobial resistance. *Diabetes Metab Syndr Clin Res Rev*. 2013;7(2):101–7.
18. Premanath R, Suresh S, Alva PP, S KA. Biofilm forming abilities of microorganisms associated with diabetic wound infection: a study from a tertiary care hospital. *Biomed Pharmacol J*. 2019;12(2):669–76.
19. Coşkun B, Ayhan M, Ulusoy S, Guner R. Bacterial profile and antimicrobial resistance patterns of diabetic foot infections in a major research hospital of Turkey. *Antibiotics*. 2024;13(7):599.
20. Moya-Salazar J, Chamana JM, Porras-Rivera D, Goicochea-Palomino EA, Salazar CR, Contreras-Pulache H. Increase in antibiotic resistance in diabetic foot infections among peruvian patients: a single-center cross-sectional study. *Front Endocrinol*. 2023;14:1267699.
21. Ahmed A, Alvi SA, Aftab IB, Akhtar F. Bacterial diversity with emerging antimicrobial resistance of diabetic foot ulceration and current detection techniques: a review. *Electron J Gen Med*. 2021;18(6):em323.
22. Mamdoh H, Hassanein KM, Eltoony LF, Khalifa WA, Hamed E, Alshammari TO, et al. Clinical and bacteriological analyses of biofilm-forming staphylococci isolated from diabetic foot ulcers. *Infect Drug Resist*. 2023;16:1737–50.
23. Neut D, Tijdens-Creusen EJ, Bulstra SK, Van Der Mei HC, Busscher HJ. Biofilms in chronic diabetic foot ulcers—a study of 2 cases. *Acta Orthop*. 2011;82(3):383–5.

Cite this article: Tahashildar M, Harakuni S, Metgud S, Kajagar BM, Togale M. Correlation of antimicrobial resistance, biofilm production of bacteria infecting grade 2 and 3 diabetic foot ulcers. *Indian J Microbiol Res*. 2025;12(3):376–380.