ORIGINAL ARTICLE

Integrated Genomic Phenotypic and Host-response Analysis of Multidrug-resistant Diabetic Foot Infections: A Tertiary Center Study from Kerala India

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Visual Abstract

Abstract

Background: Diabetic foot infections (DFIs) are a primary driver of amputation, worsened by multidrug-resistant (MDR) organisms. The link between pathogen genomics, virulence (e.g., biofilm), and host immunity is poorly quantified. This study aimed to integrate these multi-omic data streams to identify predictors of treatment failure.

Materials and methods: This prospective, single-center cohort study enrolled 150 DFI patients. Deep-tissue and autologous healthy-skin biopsies were collected. Pathogens were identified (culture/16S rRNA), with susceptibility and biofilm capacity tested. MDR isolates ($n = 76$) underwent whole-genome sequencing (WGS). Host response was quantified using an eight-gene quantitative reverse transcription polymerase chain reaction (qRT-PCR) panel, normalized to autologous controls. The primary outcome was "healing failure" (amputation or persistent ulcer) at 12 weeks. A least absolute shrinkage and selection operator (LASSO)-penalized regression model was developed and cross-validated.

Results: The final analytical cohort included 112 culture-positive patients; 68/112 (60.7%) had an MDR infection. WGS identified high-risk clones (*Escherichia coli* ST131, *Klebsiella pneumoniae* ST258). Healing failure occurred in 34/112 (30.4%) patients. Strong biofilm production (adjusted OR 3.81, 95% CI 1.92–7.55) and a high host IL-10:TNF-α expression ratio (AOR 2.98, 95% CI 1.41–6.30) were independent predictors of healing failure. The LASSO model achieved an optimism-corrected area under the curve (AUC) of 0.79 (95% CI 0.71–0.87) with acceptable calibration (Brier score: 0.18; slope: 0.95).

Conclusion: In this DFI cohort, pathogen biofilm capacity and a modulated host immune signature were independently associated with treatment failure, highlighting potential new diagnostic and therapeutic targets.

Keywords: Biofilms, Diabetic foot infection, Host-pathogen interactions, Interleukin-10, Multidrug-resistant, Predictive model, Wound healing.

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Introduction

Diabetic foot infections (DFIs) are a leading cause of nontraumatic lower limb amputations and represent a significant global health burden, particularly in regions with a high prevalence of diabetes, such as Kerala, India.[1] The management of DFIs is increasingly complicated by their frequent polymicrobial nature and the rising prevalence of multidrug-resistant (MDR) organisms.[2,3]

Current literature has extensively cataloged pathogen prevalence but often suffers from limitations.[4] Many studies are descriptive, relying on standard culture techniques that may miss fastidious organisms or anaerobes. Crucially, they often fail to integrate key pathogenic determinants, such as the genomic drivers of resistance, virulence factor profiles, and critical phenotypic states, particularly the capacity to form biofilms.[5]

Furthermore, DFI represents an interaction between the pathogen and the host. The local immune environment at the wound site—whether robust, impaired, or dysregulated—is a critical determinant of outcome. Most studies fail to characterize this response, and those that do often lack appropriate autologous control tissues, making interpretation of fold-change data difficult.

This study was undertaken to address these gaps by integrating three data streams at the patient level. The objectives were to: (1) characterize the aerobic/anaerobic bacteriological profile; (2) quantify biofilm-forming capacity as a key virulence phenotype; (3) define the genomic resistome and virulome of MDR pathogens using WGS; (4) characterize the in situ host immune-inflammatory response at the wound site relative to autologous healthy-skin controls; and (5) integrate these datasets to identify independent predictors and build a validated model for treatment failure.

Materials and Methods

Study Design and Ethical Approval

This prospective, single-center cohort study was conducted over an 18-month period (January 2023 to June 2024) at a tertiary care teaching hospital in Kerala, India. The study was approved by the Institutional Ethics Committee (IEC No. 22/IEC/SUTAMS/2023). Written informed consent was obtained from all participants.

Patient Population and Outcomes

All consenting adult (≥18 years) patients with type 2 diabetes mellitus admitted with a new or worsening DFI [per Infectious Diseases Society of America (IDSA) guidelines] were enrolled. Antibiotic use within 14 days was recorded as a covariate.

The primary composite outcome was "healing failure" at 12 weeks, defined as either (1) the need for a major amputation (below- or above-knee) or (2) a persistent, unhealed ulcer at the 12-week follow-up visit. Patients who died from unrelated causes ($n = 3$) or were lost to follow-up ($n = 8$) were excluded from the final analytical cohort. All 112 patients in the analytical cohort completed the 12-week follow-up.

Data and Sample Collection

A standardized case report form captured demographics, metabolic control (HbA1c), comorbidities (PAD, neuropathy), wound classifications (PEDIS, WIfI), and key covariates, including surgical debridement and revascularization status.

After debridement, four tissue samples were collected: (1) a deep-tissue biopsy for aerobic/anaerobic culture; (2) a second

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deep-tissue biopsy for 16S rRNA sequencing if needed; (3) a 3 mm punch biopsy from the active wound edge, placed in RNAlater; and (4) a 4 mm punch biopsy from healthy, nonacral skin (proximal thigh) of the same patient as an autologous control.

Microbiological Analysis

Tissue was homogenized and plated for aerobic and anaerobic culture. Identification was performed by standard biochemical methods or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). 16S rRNA gene sequencing was used for difficult-to-identify isolates. Antimicrobial susceptibility testing (AST) was performed per Clinical and Laboratory Standards Institute (CLSI) guidelines. MDR was defined per Magiorakos et al.[6]

For polymicrobial infections, an "index pathogen" was designated for patient-level analysis based on a prespecified algorithm: (1) the isolate with the highest level of resistance (MDR > non-MDR), and (2) in cases of equivalent resistance, the isolate with the highest semiquantitative abundance.

Advanced Pathogen Analysis

Quantitative biofilm assay: All isolates were assessed in triplicate using a 96-well plate crystal violet assay. Absorbance (OD) was read at 570 nm. Isolates were categorized relative to a nonadherent control (*Escherichia coli* ATCC 25922) cutoff (ODc): nonformer (OD ≤ ODc), weak (ODc < OD ≤ two × ODc), moderate (two × ODc < OD ≤ four × ODc), or strong (OD > four × ODc). The interassay coefficient of variation was <15%.

Whole-genome sequencing (WGS): Genomic DNA from all MDR isolates was sequenced on an Illumina NovaSeq 6000. Reads were quality-trimmed (Trimmomatic), assembled (SPAdes), and assessed for quality (QUAST). WGS libraries achieved a mean read depth of >80× and a mean N50 of >55 kb. Contamination was screened (Kraken2). Resistomes (ResFinder) and virulence factors (VFDB) were identified. Multilocus sequence typing (MLST) (v2.0) was determined.

Host Immunological Analysis

Total RNA was extracted from wound-edge and autologous skin biopsies (RNeasy kit). cDNA was synthesized, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed for a targeted eight-gene panel (TNF-α, IL-1β, IL-6, IL-10, CD4, CD68, MMP-9, TGF-β) and two reference genes (GAPDH, B2M). Relative expression was calculated using the ΔΔCt method, normalized to the geometric mean of the reference genes, and expressed as fold-change relative to the patient's own healthy skin sample. Primer efficiencies were verified (95–105%). Assay precision was high (mean SD of Ct triplicates = 0.21 Ct).

Statistical Analysis

All analyses were performed in R (v4.3.1). The primary analytical cohort consisted of 112 culture-positive patients with 12-week outcome data.

Missing data: Missingness in covariates (<5%) was handled using multiple imputation by chained equations (MICE, five imputed datasets).

Risk factor analysis: A multivariable logistic regression model was built to identify risk factors for "MDR infection" (patient-level). Variables were selected a priori (age, HbA1c, PAD, prior DFI, duration of DM). Continuous variables were modeled using restricted cubic splines.

Outcome analysis: A separate multivariable logistic regression model assessed predictors of "healing failure."

Host response: Gene expression fold-changes were $\log_2$-transformed. *p*-values for the eight-gene panel were adjusted for multiple comparisons [Benjamini-Hochberg false discovery rate (FDR)].

Predictive model: A least absolute shrinkage and selection operator (LASSO)-penalized logistic regression model was built using 22 candidate variables. Performance was validated using 10-fold cross-validation, repeated 10 times. Model optimism was assessed via 200 bootstrap resamples.

Sensitivity analyses: Six prespecified sensitivity analyses were conducted to test robustness, including modeling biofilm as a continuous variable, using "amputation only" as the outcome, and an inverse probability of censoring weighting (IPCW) analysis to assess selection bias.

Results

Patient Cohort

A total of 150 patients were enrolled. Thirty-eight were excluded (27 culture-negative, 8 lost to follow-up, 3 died of unrelated causes), leaving a final analytical cohort of 112 patients. Mean age was 62.4 ± 11.2 years, 66.2% were male, and mean HbA1c was 8.9% ± 1.4%. Baseline wound severity was high (45% WIfI stage 3 or 4).

Bacteriological Profile and Multidrug-resistant Prevalence

The 112 patients yielded 131 bacterial isolates; 58/112 (51.8%) infections were polymicrobial. Gram-negative organisms were predominant (55.0% of isolates). At the patient level, the most common index pathogens were *Staphylococcus aureus* (*n* = 25, 22.3%), *E. coli* (*n* = 21, 18.8%), and *Klebsiella pneumoniae* (*n* = 18, 16.1%) (Table 1).

Overall, 76/131 (58.0%) of isolates were MDR. At the patient level, 68/112 (60.7%) patients had an infection involving at least one MDR organism.

Genomic Analysis of Multidrug-resistant Isolates

WGS was performed on all 76 MDR isolates. This analysis confirmed the genomic basis for resistance and identified the circulation of high-risk international clones (Table 2).

Among 28 MDR *E. coli* isolates, 12 (42.9%) belonged to the ST131 clone, all carrying bla_CTX-M-15. Among 22 MDR *K. pneumoniae* isolates, 7 (31.8%) were ST258, and 3 carried bla_KPC-2. Carbapenem resistance was also driven by bla_NDM-5 (identified in 5 *K. pneumoniae* and 2 Acinetobacter baumannii). All 14 MRSA isolates were ST239-SCCmecIII, and 5 (35.7%) carried genes for the Panton-Valentine Leukocidin (pvl) toxin.

Risk Factors for Multidrug-resistant Infection

The a priori multivariable logistic regression model (Table 3) identified two significant independent predictors for having an MDR infection (*n* = 68 events): previous DFI (AOR 4.05, 95% CI 1.85–8.87) and baseline HbA1c (AOR 1.41, 95% CI 1.10–1.82). A sensitivity analysis confirmed that antibiotic use in the last 14 days was also a predictor (AOR 2.45, 95% CI 1.15–5.21) but did not meaningfully alter the main findings.

Primary Outcome and Integrated Analysis

The primary composite outcome, healing failure at 12 weeks, occurred in 34 of

Table 1: Distribution of 112 index pathogens from DFIs

Bacterial isolate (index)	*Frequency (n)*	*Percentage (%)*
S. aureus	25	22.3%
E. coli	21	18.8%
K. pneumoniae	18	16.1%
Pseudomonas aeruginosa	14	12.5%
Acinetobacter spp.	9	8.0%
Other*	25	22.3%
Total	112	100%

*Other includes: Anaerobes (*n* = 7), *Enterococcus* spp. (*n* = 6), *Proteus* spp. (*n* = 5), and other (*n* = 7)

Table 2: Key genomic characteristics of the 76 MDR isolates

Organism (n = 76)	*Common sequence types (STs) (n)*	*Key AMR genes detected (n)*	*Key virulence factors (n)*
E. coli (*n* = 28)	ST131 (12)	_bla_CTX-M-15 (20)	*iutA* (aerobactin) (15)
	ST648 (4)	*aac*(6')-*Ib-cr* (11)	*fimH* (adhesion) (26)
K. pneumoniae (*n* = 22)	ST258 (7)	_bla_KPC-2 (3)	*rmpA* (hypermucoidy) (4)
	ST11 (5)	_bla_NDM-5 (5)	*ybtS* (yersiniabactin) (9)
S. aureus (*n* = 14)	ST239 (14)	*mecA* (SCC_mec_III) (14)	*pvl* (PVL toxin) (5)
		aac(6')-*Ie* (8)	*icaA* (biofilm) (11)
P. aeruginosa (*n* = 6)	ST235 (3)	_bla_VIM-2 (2)	*lasR* (quorum sensing) (6)
Acinetobacter spp. (*n* = 6)	ST2 (4)	_bla_OXA-23 (5)	*cpaA* (adhesion) (4)

AMR, antimicrobial resistance; pvl, Panton-Valentine leucocidin; ST, sequence type

Table 3: Multivariable logistic regression model for risk factors associated with MDR infection (*n* = 112 patients, 68 MDR events)

Predictor variable	*Adjusted odds ratio (AOR)*	*95% confidence interval (CI)*	*p-value*
Prior DFI (yes vs no)	4.05	1.85–8.87	<0.001
HbA1c (per 1% increase)[1]	1.41	1.10–1.82	0.007
Peripheral arterial disease (yes vs no)	1.33	0.71–2.49	0.38
Age (per 10-year increase)[1]	1.05	0.89–1.24	0.55
Duration of diabetes (per 5 years)	1.12	0.95–1.32	0.18

[1]Modeled using restricted cubic splines. Results pooled from 5 multiply imputed datasets

Table 4: Multivariable logistic regression model for predictors of healing failure at 12 weeks (*n* = 112 patients, 34 failure events)

Predictor variable	*Adjusted odds ratio (AOR)*	*95% confidence interval (CI)*	*p-value*
Strong biofilm (vs non/weak/mod)	3.81	1.92–7.55	<0.001
IL-10:TNF-alpha ratio (per log_{2}-unit)	2.98	1.41–6.30	0.004
WIfI score (per stage increase)	2.15	1.45–3.19	<0.001
MDR status (yes vs no)	1.89	0.88–4.06	0.101
Surgical source control (achieved vs not)	0.45	0.22–0.92	0.028
HbA1c (per 1% increase)[1]	1.20	0.98–1.47	0.08

[1]Modeled using restricted cubic splines. Model adjusted for age and PAD. Results pooled from 5 multiply imputed datasets

Table 5: Clinical utility of the LASSO model at different risk thresholds (from 10-fold cross-validated predictions)

Risk threshold	*Sensitivity*	*Specificity*	*Positive predictive value (PPV)*	*Negative predictive value (NPV)*
20%	88.2% (72.6–96.7)	65.4% (53.5–76.0)	48.4% (35.6–61.4)	93.1% (83.3–97.9)
30%	70.6% (52.5–84.9)	82.1% (71.3–90.2)	58.5% (42.1–73.7)	88.4% (78.4–94.9)
40%	52.9% (35.1–70.2)	91.0% (82.4–96.4)	66.7% (46.0–83.5)	84.1% (74.4–91.3)

Thresholds for intervention based on predicted probability of healing failure. Prevalence of outcome = 30.4%. 95% confidence intervals calculated *via* the bootstrap method

112 patients (30.4%), including 22 major amputations and 12 persistent ulcers.

In the multivariable model for healing failure (*n* = 34 events, Table 4), strong biofilm production by the index pathogen was the most significant pathogen-related predictor, stronger than MDR status alone.

Host-immune Signature

After FDR correction, patients who failed to heal showed significantly higher expression of IL-10 (mean $\log_2$ fold-change 2.9, $q = 0.008$) and significantly lower expression of TNF-α (mean $\log_2$ fold-change –1.8, $q = 0.015$) compared to those who healed. The IL-10:TNF-α expression ratio was also an independent predictor of healing failure (see Table 4).

Predictive Model for Healing Failure

A LASSO-penalized regression model was built using 22 candidate variables, retaining 7 predictors. Performance, assessed by 10-times repeated 10-fold cross-validation, achieved a mean AUC of 0.81 (95% CI 0.74–0.88). The optimism-corrected AUC was 0.79 (95% CI 0.71–0.87). The model was well calibrated (mean cross-validated Brier score: 0.18, 95% CI 0.14–0.22; optimism-corrected calibration slope: 0.95, 95% CI 0.88–1.03).

Decision curve analysis showed a net benefit over default strategies. The clinical utility of the model at specific thresholds is presented in Table 5.

Sensitivity Analyses

The primary findings were robust across all six sensitivity analyses. Modeling biofilm OD as a continuous variable (AOR 1.9, $p = 0.002$) and using "major amputation only" as the outcome (AOR 3.1, $p = 0.003$) confirmed the association. The findings also held in the complete-case analysis, the polymicrobial-only cohort, when the index pathogen was defined by abundance, and in the IPCW analysis.

Discussion

This prospective study integrated pathogen genomics, virulence phenotyping, and host

transcriptomics to identify predictors of DFI treatment failure. We found that 60.7% of patients harbored an MDR organism, with prior DFI and poor glycemic control (HbA1c) being key risk factors. The WGS analysis (Table 2) confirmed that this burden is driven by high-risk, globally disseminated clones such as *E. coli* ST131 and *K. pneumoniae* ST258.

The primary finding is that while MDR status is a risk factor, it was not the strongest independent predictor of 12-week healing failure. Instead, the phenotype of the index pathogen ("strong biofilm production") and the host immune response (a high IL-10:TNF-α ratio, suggestive of a "blunted" immune state) were independent and clinically relevant predictors. This supports the hypothesis that the bacteria's ability to protect itself *via* biofilm and modulate the local immune environment is a critical pathogenic mechanism.

Our predictive model, which integrated these novel markers, achieved a robust, optimism-corrected AUC of 0.79 with good calibration. This demonstrates the potential utility of an integrated approach. However, given the borderline events-per-variable ratio (34 events/22 candidate predictors), the model coefficients may be unstable. Furthermore, the sample size provided limited precision for detecting smaller effect sizes. Therefore, these findings and the model require mandatory external validation in a separate, larger cohort before any clinical consideration.

This study has several limitations. It is a single-center study, limiting the generalizability of specific pathogen prevalences. The "index pathogen" definition, while prespecified and robust to sensitivity analyses, simplifies complex polymicrobial dynamics. We also acknowledge that by excluding 3 patient deaths, we did not formally model the competing risk of mortality. A Fine-Gray competing risk analysis was considered but was underpowered due to the small number of events.

An independent multicenter cohort validation is planned for 2025 (protocol under development) to assess transportability of the LASSO model.

Conclusion

This study demonstrates that DFI outcomes are associated with a complex triad of pathogen resistance, virulence phenotype, and host immune response. Strong biofilm production and a high IL-10:TNF-α ratio at the wound site are independent predictors of treatment failure. These findings suggest that future DFI management strategies may be improved by incorporating diagnostics that assess biofilm and host response. These findings and the proposed model must be validated in external cohorts.

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