

Repurposing Metformin to Combat Multidrug-Resistant *Proteus Mirabilis* in Diabetic Foot Ulcers: Insights into Antibiotic Resistance and Some ABC Efflux Pump Genes

Metformin
against *Proteus*
Mirabilis isolated
from Diabetic
Foot Ulcers

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ABSTRACT

Objective: To evaluate the antibiotic resistance profile, distribution of ABC efflux pump genes, and the in vitro antibacterial activity of metformin against *Proteus mirabilis* isolated from diabetic foot ulcers.

Study Design: Cross-sectional laboratory-based study

Place and Duration of Study: This study was conducted at the Department of Biology, College of Science for Women, University of Babylon, in collaboration with Al-Marjan Teaching Hospital and Al-Kafeel Specialized Hospital, Iraq, from January 2026 to April 2026.

Methods: Clinical specimens from 188 patients with diabetic foot ulcers were cultured for bacterial isolation. *Proteus mirabilis* isolates were identified using standard microbiological methods. Antibiotic susceptibility testing was performed according to CLSI guidelines. The *macA*, *macB*, and *tolC* genes were detected by PCR, while the antibacterial activity and minimum inhibitory concentration (MIC) of metformin were determined using the broth microdilution method.

Results: Of 286 bacterial isolates recovered, 30 (10.49%) were identified as *P. mirabilis*. High multidrug resistance (MARI 0.38–0.84) was observed. The *macA*, *macB*, and *tolC* genes were detected in 90%, 86.7%, and 100% of isolates, respectively. Metformin exhibited dose-dependent antibacterial activity with an MIC of 100 mg/mL.

Conclusion: MacAB-TolC efflux pumps are strongly associated with antimicrobial resistance in *P. mirabilis* isolated from diabetic foot ulcers. Metformin demonstrated promising adjunctive antibacterial activity and warrants further investigation as a potential therapeutic strategy.

Key Words: *P. mirabilis*; Diabetic foot ulcer; *macA*; *macB*; *tolC*; Metformin

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INTRODUCTION

DFUs are a serious complication of diabetes, affecting approximately 18.6 million people annually and associated with a five-year mortality rate exceeding 70%⁽¹⁾. These ulcers are often accompanied by multimicrobial infections due to factors such as weakened immunity and poor blood supply⁽²⁾.

P. mirabilis is commonly found in cultures of chronic DFUs⁽³⁾, and the therapeutic challenge is compounded by the increasing number of multidrug-resistant (MDR)

strains, contributing to a global antibiotic resistance crisis projected to cause up to 10 million deaths annually by 2050⁽⁴⁾. ATP efflux pumps play a crucial role in actively exporting antimicrobial agents and reducing intracellular drug concentrations. Overexpression of these pumps contributes to treatment failure and the emergence of MDR patterns⁽⁵⁾.

Drug repurposing can identify new antimicrobial applications for existing medications. Metformin, primarily used to treat type 2 diabetes, shows promise due to its anti-inflammatory and antibacterial effects. Research suggests it may enhance antibiotic efficacy, modulate bacterial metabolism, reduce biofilm, and boost the immune response, indicating its potential use as an adjunctive therapy against pathogens^(6,7).

Given the join between DFUs and *P. mirabilis* infection, metformin is a promising biological applicant as an adjuvant and warrants further evaluation. Particular the limited molecular data on ABC efflux pump in *P. mirabilis* from Iraqi DFU patients, the current study aimed to characterize the molecular distribution of *macA*, *macB* and *tolC* genes among these isolates, and evaluate antibacterial activity of metformin, in vitro.

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METHODS

Study design

This study included collecting 188 clinical samples from patients suffering from DFUs at Al-Marjan Teaching and Al-Kafeel Specialized Hospitals in Iraq, during the period between January 2026 to April 2026.

Bacterial identification

Wound specimens were collected from patients with DFUs. Each specimen was directly cultured onto a range of suitable media and incubated at 37°C for 18-24 h. Colonies were identified based on their morphology with initial identification using standard microbiological procedures. Final bacterial identification was confirmed using the VITEK® 2 Compact (bioMérieux, France) according to the manufacturer's instructions.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing for *P. mirabilis* was achieved using the disk diffusion technique, according to CLSI guidelines. The method included using of 13 antibiotics (Information regarding antibiotics will be provided in detail in the results section). The Multiple-Antibiotic Resistance Index (MARI) is calculated using the formula A/B, where A represents the number of antibiotics the isolate is resistant to, and B is the total number of antibiotics studied. A MARI value of 0.2 or higher indicates exposure to high-risk antibiotic sources, while a value < 0.2 indicates infrequent or no antibiotic use⁽⁸⁾.

DNA Extraction

The DNA extraction kit from Mebep Bioscience (Mebep Bioscience/China) was used to separate genomic DNA from *P. mirabilis* isolates according to the manufacturer's protocol.

Polymerase Chain Reaction (PCR)

All *P. mirabilis* isolates underwent molecular characterization by detecting the *macA*, *macB*, and *tolC* genes using specific primer pairs (Table 1). DNA amplification was achieved in a 25 µL reaction mixture comprising forward/reverse primers (10 pmol/1 µL each), template DNA (50–500 ng/4 µL), nuclease-free water (6.5 µL), and Accustart™ Taq PCR SuperMix (12.5 µL). PCR products (5 µL) were observed using electrophoresis on agarose gel, stained with a safe red dye, exposed under UV-transilluminator and photographed, the sizes of the products were determined using a 100-base pair DNA ladder^(9,10).

Determination of the Minimum Inhibitory Concentration (MIC) for Metformin

This study evaluated the MIC and efficacy of metformin using a 96-well microplate method⁽¹¹⁾. Bacterial inoculum were added to each well, with negative controls (broth only) to ensure sterility and positive controls to confirm bacterial growth in the absence of metformin. Test rows included different metformin concentrations (12.5, 25, 50, 100, and 200 mg/mL) and were incubated at 37°C for 18 h, after which the optical density (OD) was measured to determine the MIC.

RESULTS

Distribution of Bacterial Isolates Recovered from DFUs samples

Through microbiological examination of the 188 DFU samples, 286 bacterial isolates were obtained, 10.49% were of *P. mirabilis*, and 89.51% were of other bacterial species (*S. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *E. faecalis*), as shown in Figure (1).

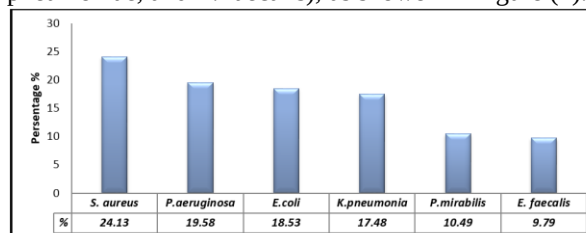


Figure No.1: Distribution of Bacterial Isolates Recovered from DFUs specimens

Resistance Profiles of *P. mirabilis*: Tests conducted on 13 antibiotics against *P. mirabilis* isolates showed that, not exhibit resistance to meropenem and levofloxacin, while resistance rates to amikacin, cefotaxime, chloramphenicol, tetracycline, doxycycline, and ceftazidime were significantly high. All strains were resistant to amoxicillin-clavulanate (Table 2).

ABC Efflux Pump Genes: The distribution of ABC efflux pump genes among the isolates showed that the *tolC* gene was present in 100% of the 30 isolates tested. Additionally, 90% of the samples were positive for the *macA* gene and 86.7% for the *macB* gene (Figure 2). Gel analysis confirms these findings; the *macA* gene was found at 255 bp, the *macB* gene at 364 bp, and the *tolC* gene amplifier at 596 bp, consistent with the lengths predicted from the primers used in the study (Figures 3).

Table No. 1: The PCR conditions and Primers sequences for genes

Primer	Sequence(5' - 3')	Product (bp)	Annealing (°C)	Ref.
MacA	F: GCACAACAAGCACCGAACAT R:CATATCCAGCCGCAGCAAAC	255	58.3°C	(12)
MacB	F: GCGTGAGCGGCGATTATTTT R: AAACGCGTGAGTTGCTGTTC	364	57.2°C	
TolC	F: CGATCGTGATGCTGCCTTTG R: GGTTCGTTTTTCGGCTTCT	596	58.3°C	

Table No. 2: Antibiotic resistance patterns of *P. mirabilis* (n=30)

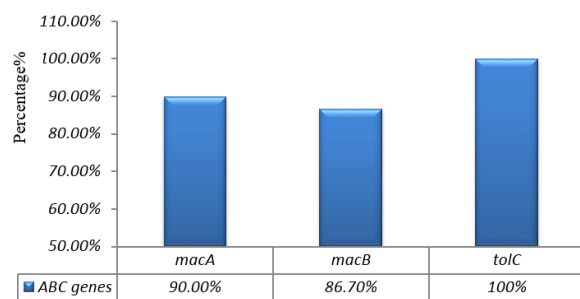
Antibiotics agents	Sensitive (n/%)		Resistance (n/%)		Total (n/%)
TE/Tetracycline 30 µg	1	3%	29	97%	30 (100%)
DXT/Doxycycline 30 µg	1	3%	29	97%	30 (100%)
CAZ/ ceftazidime 30 µg	1	3.3%	29	97%	30 (100%)
CTX/Cefotaxime 30 µg	2	7%	28	93%	30 (100%)
CN/gentamicin 10 µg	13	43%	17	57%	30 (100%)
AK/amikacine 30 µg	5	17%	25	83%	30 (100%)
LEV/levofloxacin 5 µg	27	90%	3	10%	30 (100%)
OFX/ ofloxacin 5 µg	26	87%	4	13%	30 (100%)
AMC/Amoxicillin-clavulanate 20/10 µg	0	0%	30	100%	30 (100%)
ATM/ Aztreoname 30 µg	24	80%	6	20%	30 (100%)
C/Chloramphenicol 30 µg	2	7%	28	93%	30 (100%)
IPM/ imipenem 10 µg	24	80%	6	20%	30 (100%)
MEM/meropenem 10 µg	29	97%	1	3%	30 (100%)

The MARI revealing resistance levels ranging from 0.38 to 0.84 (Table 3). All isolates (100%) had MARI values > 0.2.

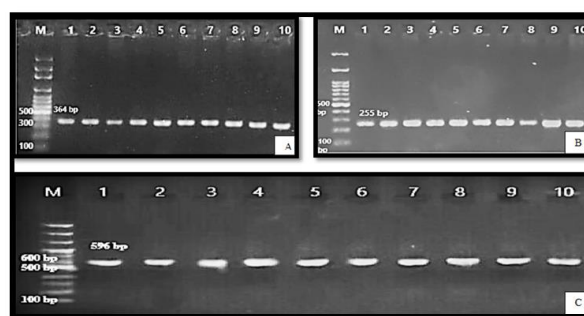
Table No.3: Multiple antibiotic resistance index values of *P. mirabilis* isolates (n=30).

Isolate code	MARI	n (%)
PM 3	0.38	1 (3.3)
PM 2, PM 6, PM 12, PM 28	0.46	4 (13.4)
PM1, PM4, PM5, PM9, PM17-23, PM27	0.53	12 (40)
PM7, PM10, PM13, PM15, PM16, PM24-26, PM 30	0.61	9 (30)
PM29	0.64	1 (3.3)
PM11, PM14	0.76	2 (6.7)
PM8	0.84	1 (3.3)

PM, *P. mirabilis*

**Figure No.2: Percentage of ABC efflux pump genes in *P. mirabilis*.**

The study demonstrated a strong association between antibiotic resistance in *P. mirabilis* and the *macA*, *macB*, and *tolC* genes, with detection rates ranging from 75% to 100%. The *tolC* gene was present in all resistance groups (100%). The significance of these results was high ($P < 0.0001$), indicating stable gene expression among isolates. However, no significant changes in gene distribution were observed among the different antibiotic resistance classes ($P = 0.818$ to 1.000).

**Figure No.3: Agarose gel electrophoresis of A; *macB* (364bp), B; *macA* (255bp), C; *tolC* (596bp) genes amplicon.**

Determination of Metformin Minimum Inhibitory Concentrations (MIC): This study investigated the effect of metformin on the growth of ten MDR *P. mirabilis* isolates at concentrations ranging from 12.5 to 200 mg/ml, using OD at 625 nm (Figure 4). The results indicate inhibition of bacterial growth in dose-dependent manner: at positive control: Maximum growth ($OD_{625} \approx 1.34-1.52$), at 12.5 mg/ml: Moderate inhibition ($\approx 0.82-0.97$). At 25 mg/ml: Further

reduction ($\approx 0.58-0.74$), at 50 mg/ml: Significant inhibition ($OD_{625} \approx 0.23-0.44$), at 100 mg/ml: Strong inhibition ($\approx 0.18-0.30$). At 200 mg/ml: no growth was recorded ($\approx 0.05-0.07$), equal to negative control. The MIC was found to be 100 mg/ml after sub- culturing.

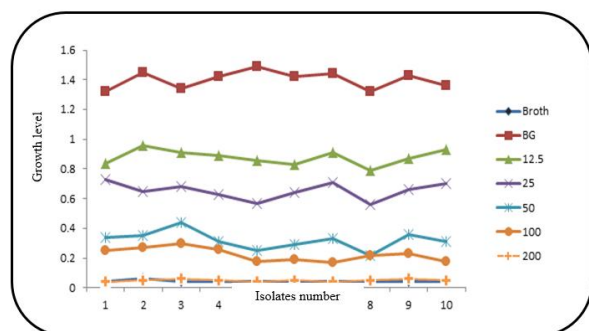


Figure No.4: Result of OD to bacterial growth with different concentration of metformin (12.5-200 mg/ml) at 625 nm. Control (BG): growth of *P.mirabilis* without drug. (n= 10)

DISCUSSION

The bacterial profile of current study is consistent with findings from studies conducted in Iraq, where *S. aureus* emerged as the most frequently isolated bacterial species, followed by *P. mirabilis* and then *E. coli*, indicating a similar distribution of pathogens in DFUs⁽¹²⁾. This aligns with the recommendations of the International DF Working Group/Infectious Diseases Society of America (IDWA), which classifies *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *P. mirabilis* as common pathogens in moderate to severe DFUs⁽¹³⁾. The high proportion of isolated Gram-negative bacteria may be attributed to chronic ulceration, repeated antibiotic therapy, prolonged hospital stays, and compromised patient immunity. It has been observed that *P. mirabilis* carries many virulence genes with resistance factors, and identified as one of the predominant Gram-negative pathogens associated with DFUs worldwide^(14, 15).

The current study indicate resistance rates ranging from 3 to 100%, characterizing these isolates with MDR phenotype, which is particularly concerning in DFU infections treated without prior culture⁽¹⁶⁾. Notably, complete resistance to amoxicillin-clavulanate and tetracyclines, along with 57% resistance to gentamicin, possibly due to specific aminoglycoside-modifying enzymes is consistent with previous findings in Iraqi clinical settings⁽¹⁷⁾. Carbapenemase-producing *P. mirabilis* have been reported regionally in cases of DFUs, with non-producing strains achieving reduced susceptibility through overexpression of the efflux pumps⁽¹⁸⁾.

The high values of MARI suggesting significant selective pressure resulting from inappropriate or prolonged antibiotic use confirming their MDR nature. This aligns with previous research on DFUs, which

highlighted elevated MARI and MDR resulting from intensive antibiotic exposure⁽¹⁹⁾. Current international guidelines recommend that antibiotic treatment for DFU infections be based on culture and susceptibility testing to enhance treatment efficacy and reduce the spread of antibiotic resistance⁽²⁰⁾. Antibiotic resistance is meaningfully associated with efflux pumps, which showed high prevalence rates in this study, consistent with local Iraqi evidence. At the international level, genomic characterization of the *P. mirabilis* SCDR1 strain revealed the co-occurrence of the MacAB-TolC pumps, which prone to overexpression under sustained antibiotic pressure. Our study demonstrated that ABC antibiotic ejection pump genes are broadly expressed across different classes of antibiotics; no specific antibiotic resistance pattern significantly affected their gene expression. The genes responsible for antibiotic resistance in *P. mirabilis* are essential for its survival under the stressful conditions of diabetic foot. The absence of the MacA, MacB, and tolC genes impairs the formation of a macrolide-specific export system, as supported by genomic data⁽²⁰⁾.

CONCLUSION

Current research underscores the importance of *P.mirabilis* in DFUs and calls for local antibiotic treatment guide in Iraq. It also highlights a research gap, noting that many studies focus solely on antibiotic susceptibility, neglecting other non-antibacterial agents such as metformin. The study suggests a reassessment of metformin as an adjunctive antimicrobial therapy and urges further research into its molecular interactions with antibiotics and their combined efficacy in patient care.

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Author's Contribution:

Concept & Design or acquisition of analysis or interpretation of data:	Nahida Fitnan Ghadban, Hawraa Wahab Al-Kaim
Drafting or Revising Critically:	Nahida Fitnan Ghadban, Hawraa Wahab Al-Kaim
Final Approval of version:	All the above authors
Agreement to accountable for all aspects of work:	All the above authors

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