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Research Article

Assessment of Biofilm Formation Ability and the Presence of *mrpA* and *rsbA* Genes in *Proteus mirabilis* isolated from Diabetic Wounds

Maryam Fatemi[1], Zahra Hejazi[1], Roya Moravej*[2]

1- Department of Biology, Ma. C., IslamicAzadUniversity, Mashhad, Iran.
2- Department of Biology, Ma. C., Islamic Azad University, Mashhad, Iran.

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ABSTRACT

Proteus mirabilis is a clinically significant opportunistic pathogen that harbors the *mrpA* and *rsbA* genes, which are pivotal in mediating biofilm formation and bacterial adhesion to host surfaces. Diabetic wounds, a prevalent complication of diabetes mellitus, pose substantial challenges to patient health and quality of life due to their susceptibility to persistent infections. This study aimed to evaluate the biofilm-forming capacity and the presence of *mrpA* and *rsbA* genes in *P. mirabilis* isolates from diabetic wounds. A total of 150 wound samples (69 females and 81 males) were collected from Mashhad Pathobiology Laboratory between July and March 2024. Isolates were identified using conventional microbiological and biochemical assays, followed by PCR confirmation. Biofilm formation was assessed through Congo Red Agar and microtiter plate assays, while the presence of *mrpA* and *rsbA* was determined by gene-specific PCR. Out of 150 samples, 17 isolates (11.3%) were confirmed as *P. mirabilis*. Phenotypic analysis indicated that 8 isolates were strong biofilm producers, 7 moderate, and 2 weak. Notably, all isolates (100%) carried both *mrpA* and *rsbA* genes. These findings highlight the integral role of *mrpA* and *rsbA* in biofilm development in diabetic wound-derived *P. mirabilis* strains. Insights into these genetic determinants may inform the design of targeted interventions to prevent and control biofilm-associated infections, ultimately improving therapeutic outcomes in patients with diabetic wounds.

1. Introduction

Diabetes mellitus is one of the most common chronic metabolic disorders worldwide and is associated with severe complications, including diabetic wounds (Elhoshi *et al*., 2023). In Iran, approximately eight million people live with diabetes, and about 25% of them nearly two million individuals develop diabetic wounds during their lifetime. Each year, around 50,000 diabetic patients experience chronic wounds, highlighting the importance of early care and regular follow-up to prevent complications (Hadidi *et al*., 2014). Diabetic wounds are highly susceptible to infection because of reduced blood flow, neuropathy, and impaired immune function (Cestari *et al*., 2013). Among the pathogens involved, *Proteus mirabilis* is considered an important opportunistic bacterium. Its pathogenicity is mainly related to its ability to produce urease, exhibit swarming motility, and form biofilms (Nissanka *et al*., 2025). Biofilms provide structural protection for bacteria, making them more resistant to antibiotics and host immune defenses, which complicates treatment and often leads to chronic

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*Corresponding author: Roya Moravej
E-mail address: Moravej82@iau.ir

infections (Kynshi *et al*., 2025; Elhoshi *et al*., 2023). Two virulence-associated genes, manose-resistant Proteus-like fimbriae (*mrpA*) and regulator of sigma factor B (*rsbA*), play critical roles in these processes (Sayal *et al*., 2018). The *mrpA* gene encodes mannose-resistant *Proteus* fimbriae, which enhance bacterial adhesion and contribute to biofilm formation and swarming motility (Thabit *et al*., 2025). The *rsbA* gene acts as a regulator influencing swarming behavior and the production of extracellular polysaccharides, both of which are essential for biofilm development (Elhoshi *et al*., 2023). Several studies have reported strong correlations between these genes and both biofilm strength and multidrug resistance in clinical isolates (Al-Dulaimy *et al*., 2023). Infections caused by *P. mirabilis* in diabetic wounds can lead to serious complications, such as tissue necrosis, foul odor, deep tissue involvement, and even osteomyelitis (Elhosseini *et al*., 2024). Despite this, most studies on diabetic wound infections have focused on more common pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and only limited research has examined *P. mirabilis* in this context. The present study was designed to assess the biofilm-forming ability and the presence of *mrpA* and *rsbA* genes in *Proteus mirabilis* isolates obtained from diabetic wounds. These findings may help improve understanding of virulence mechanisms and support better strategies for the management and treatment of these infections.

2. Materials and Methods

2.1. Collection and Identification of isolates

This study was designed as a laboratory-based experimental investigation. A total of 150 suspected diabetic wound samples (69 females and 81 males) were collected from the Pathobiology Laboratory in Mashhad between July and March 2024. The samples were transported under sterile conditions to the Microbiology Laboratory of the Faculty of Basic Sciences, Azad University, Mashhad. Clinical specimens (approximately 2–3 mL each) were transported to the laboratory within 2 hours of collection under sterile conditions in screw-capped containers at 4 °C to maintain bacterial viability. Initial isolation and identification of *Proteus mirabilis* were performed using standard differential and biochemical tests, including colony morphology, Gram staining, culture on Eosin Methylene Blue (EMB) agar, citrate utilisation, motility test, and Triple Sugar Iron (TSI) test (Sharifian *et al*., 2023). All media and reagents were purchased from Merck, Darmstadt, Germany. Final confirmation of *P. mirabilis* was carried out using PCR. It is noteworthy that this study was laboratory-based and experimental, and was conducted following approval by the Ethics Committee of Mashhad University of Medical Sciences (IR.IAU.MSHD.REC.1403.064).

2.2. Molecular Identification of Proteus mirabilis

Clinical isolates that exhibited biochemical profiles consistent with *Proteus mirabilis* were confirmed through molecular identification targeting the *16S rRNA* gene.

2.2.1. DNA Extraction

Genomic DNA was extracted using the boiling method. The purity and concentration of extracted DNA were measured with a NanoDrop spectrophotometer (Abadi *et al*., 2018).

2.2.2. PCR and Primer Design

Gene-specific primers were designed based on sequences retrieved from NCBI and validated via BlastN. Analytical sensitivity was evaluated through ten-fold serial dilutions of genomic DNA extracted from standard strains including *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853. The limit of detection (LOD) was estimated to be approximately 100 bacterial cells per reaction. Positive controls consisted of genomic DNA from confirmed *P. mirabilis* (ATCC 43071) obtained from the Persian Type Culture Collection (PTCC), Iran. PCR-grade water was used as the negative control. The details of the primers used in this study are presented in Table 1. PCR reactions (25 µL) included: 12.5 µL of 2× PCR master mix (including Taq polymerase, dNTPs, $MgCl_2$, and buffer), 2 µL of template DNA, 1 µL of each forward and reverse primer, and 8.5 µL of nuclease-free distilled water. Thermal cycling conditions were: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 30 s; followed by a final extension at 72°C for 7 min. The same program was applied for all three target genes.

2.2.3. Gel Electrophoresis

PCR products were resolved on 1.5% agarose gel prepared by dissolving 0.9 g of agarose powder in 60 mL of 1× TAE buffer, with Green Viewer at 80 V for 60 min, visualized under UV illumination, and documented using a gel imaging system. A molecular weight marker (DNA ladder) was included in each run to estimate product sizes.

2.3. Phenotypic Assessment of Biofilm Formation

Biofilm production among confirmed *P. mirabilis* isolates was evaluated by two methods: the Congo Red Agar (CRA) assay and the microtiter plate assay (MTP).

2.3.1. Congo Red Agar (CRA) Assay

The CRA method was employed to evaluate biofilm production in *Proteus mirabilis* isolates. Briefly, 8 g of Congo Red indicator was dissolved in 1 L of distilled water and autoclaved. Subsequently, 10 g of Brain Heart Infusion (BHI) agar and 50 g of sucrose were added to the medium. The prepared media were stored at 4°C until use. A single colony from chocolate agar was picked using a sterile loop and streaked onto CRA plates. After incubation, colony morphology was assessed (Jamshidian *et al.*, 2020).

2.3.2. Microtiter Plate Assay

For this assay, 24-hour cultures of the isolates were prepared in Mueller-Hinton broth supplemented with 1% glucose. Then, 100 µL of bacterial suspension was inoculated into each well of a 96-well microtiter plate. After incubation, the supernatant from each well was removed, and the wells were washed 3–5 times with sterile phosphate-buffered saline (PBS). To fix the biofilm, 200 µL of 96% ethanol was added to each well for 15 minutes and then discarded. After air-drying, 200 µL of 1% crystal violet solution was added to each well. Following 15 minutes of staining, the dye was washed off with running water, and the plates were air-dried. Finally, 200 µL of 33% glacial acetic acid was added to each well to solubilize the dye, and optical density (OD) was measured at 492 nm using an ELISA reader. The experiment was performed in triplicate, and the mean and standard deviation of negative control wells were calculated (Nosrati *et al.*, 2017). Biofilm formation was categorized as follows:

Negative biofilm: OD ≤ ODC

Weak biofilm: ODC ≤ OD ≤ 2×ODC

Moderate biofilm: 2×ODC ≤ OD ≤ 4×ODC

Strong biofilm: 4×ODC ≤ OD

Table1. Specifications of the primers used for PCR

Target Gene	Primers Sequences 5′–3′	Amplicon Size (bp)	Reference
16SrRNA-F *16SrRNA-R*	AGATCTGATGGGTTGTCAGGT CCTGCCCATCAAGAAACGGA	161	(Alipour *et al.*, 2016)
mrpA-F *mrpA-R*	GTTGTTGCGGGTTCTGCTTT GTTTTGAGCAGCACTTGGG	366	(Al-Yasseen& Kamel., 2020)
rsbA -F *rsbA -R*	TTGAAGGACGCGATCAGACC ACTCTGCTGTCCTGTGGGTA	467	(Ahmadi *et al.*, 2014)

2.4. Molecular Detection of Biofilm-Associated Genes

The biofilm-forming potential of confirmed *P. mirabilis* isolates was assessed by detecting the *mrpA* and *rsbA* genes using PCR. DNA extraction was performed as described above. PCR amplification for both genes was conducted under similar conditions to those used for *16S rRNA*, except that the annealing temperatures were optimized for each primer set. PCR products were analyzed by electrophoresis on 1.5% agarose gel, stained with Green Viewer, and visualized under UV illumination. Positive controls included standard *P. mirabilis* strains carrying the target genes, while negative controls contained nuclease-free water (Al-Yasseen& Kamel., 2020).

2.5. Statistical analysis

Statistical analysis was performed using SPSS software (version IF027). Descriptive statistics were applied to summarize the data. The Kruskal–Wallis non-parametric test was used to compare the optical density values obtained from the biofilm formation assay. Statistical significance was considered at P ≤ 0.05.

3. Results

3.1. Phenotypic and Genotypic Characterization of Proteus mirabilis Isolates

Biochemical analysis (Table 2) revealed that, among 150 diabetic wound specimens, 128 were positive for bacterial infection, of which 17 isolates were identified as *Proteus mirabilis*. The remaining 109 isolates belonged to other bacterial species. To achieve definitive confirmation, molecular identification was performed through polymerase chain reaction (PCR) targeting the *P. mirabilis*-specific *16S rRNA* gene. Representative PCR amplification results are presented in Figure 1. Among all collected samples, 17 isolates (11.3%) were unequivocally confirmed as *P. mirabilis* by the molecular approach.

3.2. Biofilm Formation Assessment by Congo Red Agar Method

The ability of *Proteus mirabilis* isolates to form biofilms was evaluated using the Congo red agar (CRA) method. Colony morphology on CRA plates was used to categorize the isolates into three groups: black colonies with dry, rough edges were considered strong biofilm producers; red or pink colonies with rough edges were classified as moderate biofilm producers; and smooth pink colonies were considered non-biofilm producers. Among the 17 isolates tested, 8 isolates (47.05%) were identified as strong biofilm producers, 7 isolates (41.17%) as moderate producers, and 2 isolates (11.76%) as weak producers. (Table 3)

Table 2. Representative Biochemical Characteristics of a *Proteus mirabilis* Isolat in this study

Isolates	Gram Stain	Urease	Citrate	Motility	Indole	H2S	TSI	MR	VP
P. mirabilis	Negative	+	+	+	+	+	K/A	+	-

Note: Representative biochemical characteristics of a typical *P. mirabilis* isolate are shown. All 17 isolates exhibited similar results. (MR: Methyl Red, VP: Voges-Proskauer)

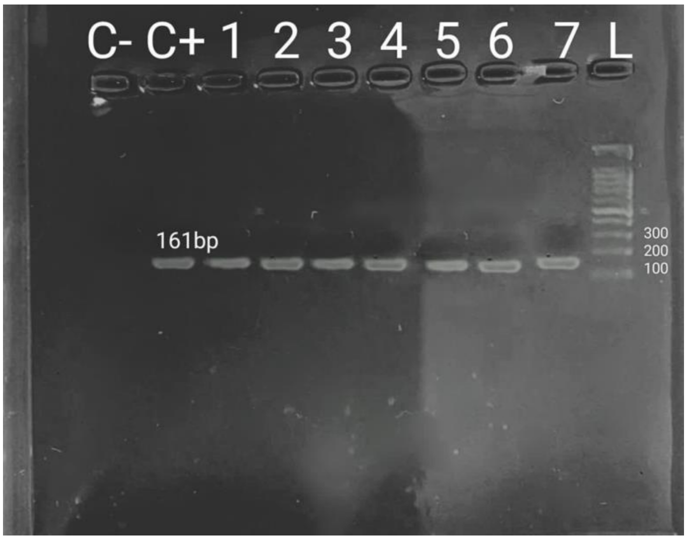

Figure 1. Electrophoresis of PCR products for *Proteus mirabilis* isolates. C-: negative control; C+: positive control; Wells 1–7: *P. mirabilis* isolates showing amplification of the 16S rRNA gene (161 bp); L: DNA ladder (100 bp).

Table 3. Biofilm formation ability of *Proteus mirabilis* isolates by Congo Red Agar (CRA) method

Biofilm formation category	colony morphology description	Number of isolates (n=17)	Percentage (%)
Strong biofilm	Black colonies with rough edges	8	47.05
Moderate biofilm	Red/pink colonies with rough edges	7	41.17
Weak / Non-biofilm	Pink colonies with smooth surface	2	11.76

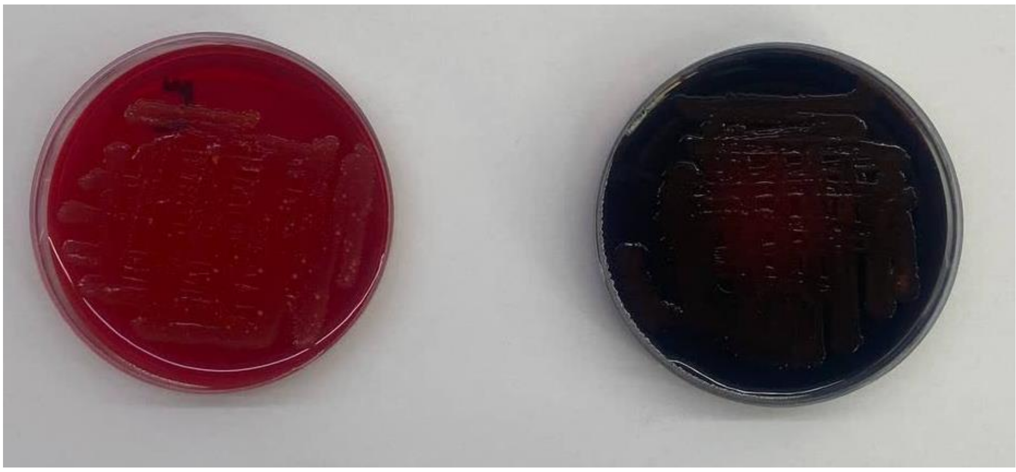

Figure 2. Congo red agar assay for biofilm detection in *Proteus mirabilis*. Isolate No. 14 (left) shows moderate biofilm formation, while Isolate No. 5 (right) exhibits strong biofilm formation.

3.3. Biofilm Formation Assessment by Microtiter Plate Method

The biofilm-forming ability of all *Proteus mirabilis* isolates was further evaluated using the microtiter plate assay. Based on the OD values, the isolates were classified into three categories: strong biofilm producers (8 isolates, 47.05%), moderate biofilm producers (7 isolates, 41.17%), and weak biofilm producers (2 isolates, 11.76%).

These findings were consistent with the results obtained from the Congo red agar method. Figure 3 illustrates the comparison of mean OD values among the 17 clinical isolates. For statistical analysis, the Kruskal-Wallis non-parametric test was applied to compare the mean OD values across the groups, considering a 95% confidence interval and a significance level of α = 0.05. The results demonstrated a statistically significant difference between the groups ($p < 0.05$), as summarized in Table 3.

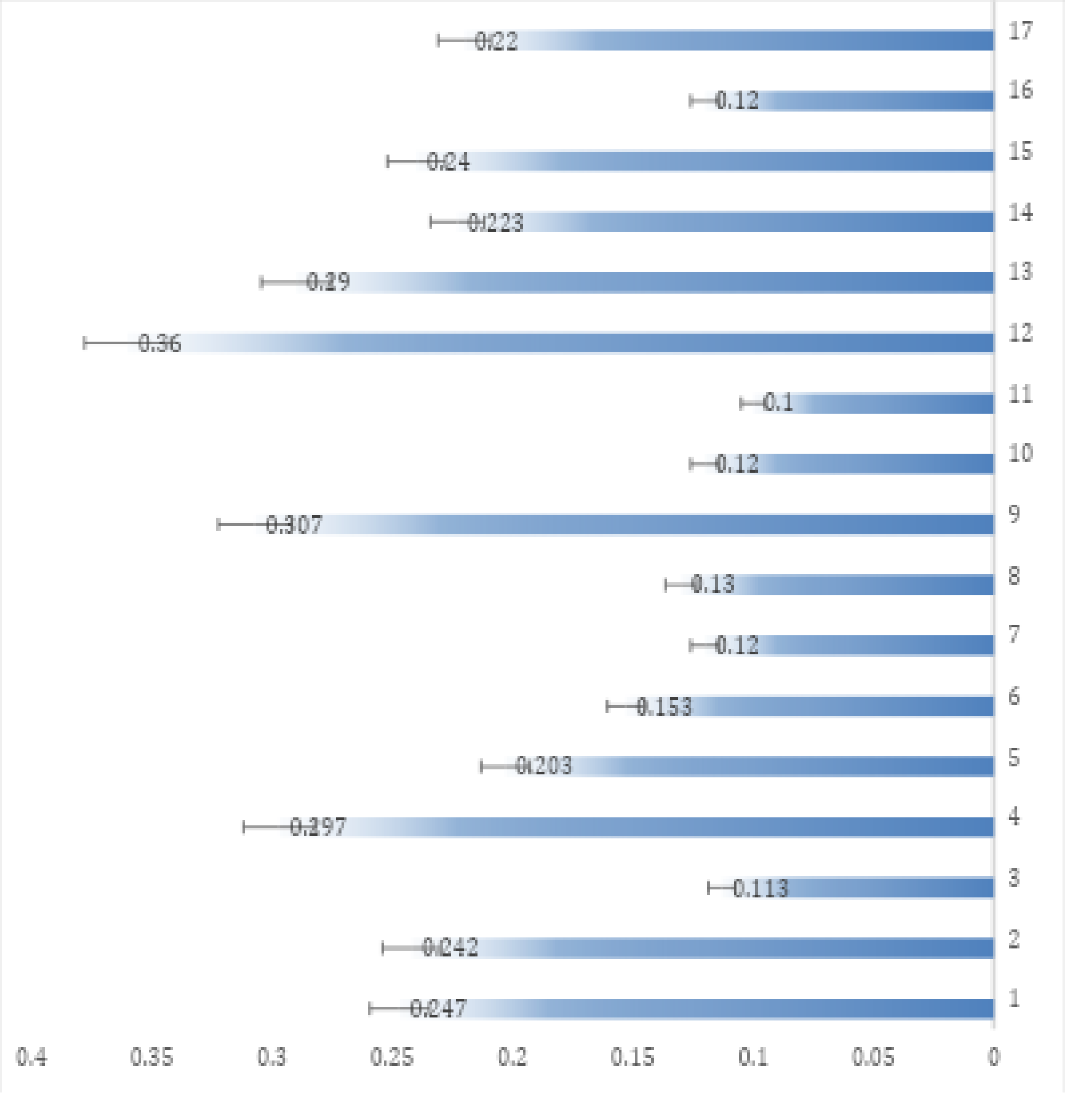

Figure 3. Comparison of optical density (OD) values for biofilm formation among 17 clinical isolates of *Proteus mirabilis* using the microtiter plate assay. Bars represent mean OD values for each isolate, indicating variation in biofilm-forming ability (strong, moderate, weak) among the tested isolates.

Table 3. Comparison of Mean Optical Density of Biofilm Formation among Clinical Isolates of *Proteus mirabilis* Using Kruskal-Wallis Test

Statistic	Chi-Square	df	p-value
Kruskal-Wallis	53.245	16	0.014

Interpretation: The p-value (< 0.05) indicates a statistically significant difference in biofilm formation among the studied groups.

3.4. Comparison of Biofilm Detection Results Using Congo Red Agar and Microtiter Plate Assays

A comparison between the Congo Red Agar method and the microtiter plate assay revealed a consistent pattern in biofilm formation among the *Proteus mirabilis* isolates. In both methods, the highest proportion of isolates exhibited strong biofilm formation (8 isolates, 47.05%), followed by moderate biofilm formers (7 isolates, 41.17%) and weak biofilm formers (2 isolates, 11.76%). This consistency indicates that both methods are reliable for phenotypic assessment of biofilm formation in *P. mirabilis* isolates. However, the nonparametric Kruskal–Wallis test, based on the optical density measurements obtained from the microtiter plate assay, demonstrated a statistically significant difference among the studied groups ($p < 0.05$). These findings suggest that the microtiter plate method not only provides a quantitative evaluation of biofilm production but also offers greater accuracy for statistical comparisons compared to the Congo Red Agar method.

3.5. Detection of mrpA and rsbA Genes for Biofilm Identification by PCR

Among the 17 identified *Proteus mirabilis* isolates, all (100%) carried the *mrpA* and *rsbA* genes. These PCR results were compared with the biofilm formation assays (Congo red and microtiter plate methods), and in all isolates that exhibited biofilm formation by these methods, the presence of *mrpA* and *rsbA* genes was confirmed. PCR products of these genes were visualized on 1.5% agarose gel electrophoresis, as shown in Figures 4 and 5.

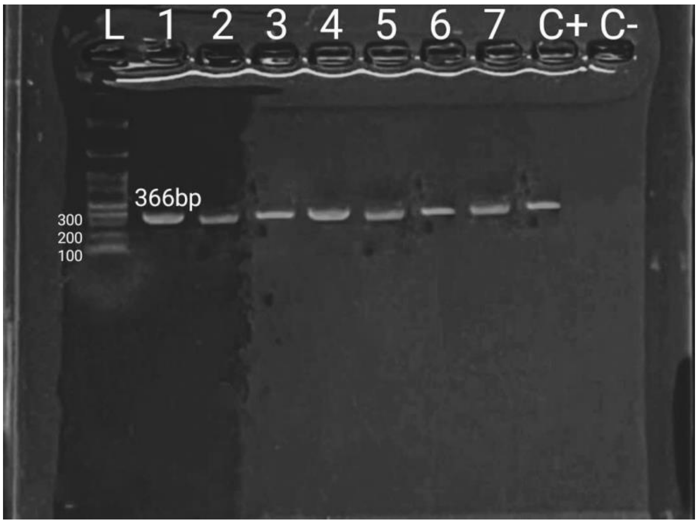

Figure 4. Gel electrophoresis of *mrpA* gene PCR products for 7 isolates of *Proteus* (L: Leder, C+: positive control, C-: negative control, Wells 1 -7 of clinical isolates)

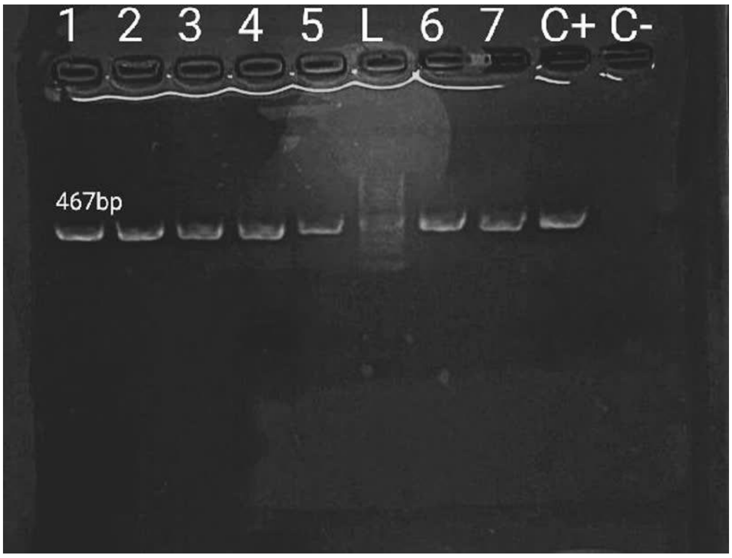

Figure 5. Gel electrophoresis of *rsbA* gene PCR products for 7 isolates of *Proteus* (L: Leder, C+: positive control, C-: negative control, Wells 1 -7 of clinical isolates)

4. Discussion

In this study, we aimed to assess the biofilm-forming ability of *Proteus mirabilis* isolates from diabetic wounds and to determine the presence of the biofilm-associated genes, *mrpA* and *rsbA*, to better understand their role in biofilm development. Molecular identification of *Proteus mirabilis* isolates using the *16S rRNA* gene successfully confirmed all biochemically characterized isolates. These results demonstrated complete concordance with the biochemical assays, thereby validating the reliability and accuracy of both methodologies for the precise identification of clinical isolates. Similar findings have been reported in previous studies, which also highlighted the robustness of *16S rRNA*-based molecular assays for differentiating P. mirabilis from closely related species within the *Enterobacteriaceae* family (Ahmadi *et al*., 2014; Alipour *et al*., 2016).

In the present study, 11.3% of diabetic wound isolates were identified as *P. mirabilis*, a prevalence consistent with prior reports indicating its moderate yet clinically significant occurrence in such infections (Al-Dulaimy *et al*., 2023). Nevertheless, variations in reported prevalence have been observed across different geographic regions, patient cohorts, and sampling methodologies. For instance, Thabit et al. (2025) documented a 14% prevalence of *P. mirabilis* in diabetic wound specimens from a Middle Eastern cohort, whereas Nissanka et al. (2025) reported a 9% prevalence in a South Asian population. These discrepancies underscore the considerable influence of regional, environmental, and clinical factors on the distribution of this pathogen.

Phenotypic analyses revealed that 47.05% of isolates were robust biofilm producers, while 41.17% exhibited moderate biofilm-forming capacity. These results align with previous studies that have identified biofilm formation as a prevalent virulence trait among clinical *P. mirabilis* isolates (Thabit *et al*., 2025; Kynshi *et al*., 2025). Notably, the proportion of strong biofilm producers observed in this study slightly exceeds the 40% reported by Elhoshi et al. (2023) in urinary tract isolates, suggesting that wound-derived strains may possess enhanced biofilm-forming potential, likely influenced by the unique environmental stressors present within diabetic tissue. Such findings highlight the necessity of assessing biofilm formation within the specific clinical context of diabetic wounds, as virulence expression may differ considerably from other infection sites.

Biofilms confer multifaceted advantages to bacteria, including augmented adhesion, protection against host immune defenses, and elevated tolerance to antimicrobial agents, collectively contributing to chronicity and presenting substantial therapeutic challenges in the management of diabetic wound infections (Elhoshi *et al*., 2023). The microtiter plate assay employed in this study demonstrated high efficacy for quantitative biofilm assessment, revealing statistically significant differences among isolates ($p < 0.05$) and providing a more precise evaluation relative to qualitative methodologies such as the Congo Red Agar assay (Nosrati *et al*., 2017). Comparable trends have been reported in prior research, wherein quantitative assays afforded superior discrimination of biofilm-forming capacity compared to agar-based phenotypic methods (Jamshidian *et al*., 2020).

At the molecular level, all isolates harbored the *mrpA* and *rsbA* genes, which encode critical factors governing fimbrial adhesion, swarming motility, and extracellular polysaccharide synthesis—processes integral to the establishment of robust biofilms (Sayal *et al*., 2018; Elhoshi *et al*., 2023). The observed concordance between phenotypic biofilm formation and the presence of these virulence genes substantiates findings from Al-Yasseen & Kamel (2020) and Thabit et al. (2025), who reported strong correlations between these genetic determinants and biofilm-forming capacity in clinical isolates from both urinary tract and wound infections. Interestingly, in comparison to urinary isolates, wound-derived strains in this study exhibited consistently high expression of *mrpA* and *rsbA*, suggesting that the wound microenvironment may preferentially select for or induce the activation of biofilm-related genetic pathways.

Clinically, infections caused by biofilm-producing *P. mirabilis* in diabetic wounds are associated with persistent inflammation, delayed wound healing, and severe complications, including tissue necrosis and osteomyelitis (Elhosseini *et al*., 2024).

Consistent with previous research, these findings underscore the considerable challenges inherent in managing biofilm-associated infections and highlight the imperative for early detection and targeted therapeutic intervention. The results of this study further reinforce that molecular screening for *mrpA* and *rsbA* genes can serve as a reliable predictor of virulence potential, complementing phenotypic assays. Compared to approaches reliant solely on phenotypic characterization, integrating molecular diagnostics affords a more accurate assessment of pathogenic potential and can inform the development of personalized, evidence-based treatment strategies for the management of diabetic wound infections.

Conclusion

This study demonstrates that all *Proteus mirabilis* isolates from diabetic wounds possess biofilm-forming capability and universally harbor the virulence genes *mrpA* and *rsbA*, which are critical for biofilm development. Environmental factors, including acidic conditions, contact with medical or biological surfaces, and subinhibitory antibiotic exposure, may further enhance gene expression and biofilm formation. These characteristics likely contribute to persistent infections in patients with compromised immune systems. These findings highlight the importance of integrating phenotypic and molecular assessments for rapid identification of high-risk strains and for guiding targeted therapeutic interventions. Future research should focus on the antibiotic resistance profiles of *P. mirabilis* isolates and the identification of effective antibacterial agents. Such efforts are essential to prevent severe complications, including deep wounds, extensive abscesses, osteomyelitis, and potential limb amputation, ultimately improving clinical outcomes for diabetic patients.

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Conflict of interest

The authors declare no conflict of interest related to this study.

Refereces

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