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Phenotypic and Genotypic Characterization of Bacteria from Diabetic Ulcers: Insights into Resistance and Biofilm Formation

Andi Ernawati, Iman Rusmana*, Aris Tri Wahyudi, Sri Murtini, and Sri Budiarti

Abstract

Diabetic ulcers are prone to colonization by multidrug-resistant bacteria, complicating treatment and recovery. Therefore, understanding the microbial profile and resistance mechanisms is essential for effective clinical management. This study isolated and characterized bacteria from diabetic ulcer patients in West Java, Indonesia. Identification was performed using the selective differential medium test and 16S rRNA sequencing. Antibiotic susceptibility was assessed via the disc diffusion method, and biofilm formation was evaluated using crystal violet staining. PCR amplification targeted blaTEM and sul1 resistance genes. A total of 15 bacterial isolates were identified, predominantly *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Six isolates exhibited a multiple antibiotic resistance (MAR) index >0.4 and demonstrated biofilm-forming capacity. The PCR result confirmed the presence of blaTEM and sul1 genes in three isolates. The findings highlight the prevalence of multidrug-resistant, biofilm-forming bacteria in diabetic ulcers, underscoring the need for targeted antimicrobial strategies and resistance monitoring.

Keywords: antibiotic resistant, biofilm, blaTEM, diabetic ulcer, gen detection, multiple antibiotic resistance (MAR) index, sul1

1. INTRODUCTION

Diabetic ulcers are a major complication of diabetes mellitus, posing substantial treatment challenges and leading to non-traumatic amputations that can be fatal [1]. Inadequately regulated blood glucose levels are prevalent in individuals with diabetic ulcers. The most common location for diabetic ulcers is foot ulcers [2]. The worldwide prevalence of diabetic foot ulcers (DFU) is approximately 6.3% among individuals with diabetes, with significant geographical variations: North America reports the highest frequency at 13%, whereas Oceania records the lowest at 3%. Europe, Asia, and Africa have prevalence rates of 5.1%, 5.5%, and 7.2%, respectively. The lifetime risk of acquiring a DFU is between 15% and 34% for those with diabetes, whereas the annual incidence ranges from 2% to 6% [3]. The prevalence of DFU in Indonesia differs by context,

with hospital studies indicating rates between 7.3% and 12% among patients with type 2 diabetes, and community estimates reaching up to 24% [2][4][5].

Diabetic ulcers often leading to chronic wounds and increased risk of infection. These wounds are frequently colonized by multidrug-resistant bacteria, which complicate treatment and prolong healing. Typical microorganisms present in infected diabetic wounds include *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Escherichia coli* [6]-[9]. *S. aureus* secretes serine proteases (V8/SspA), cysteine proteases (staphopain A/ScpA and B/ SspB), metalloproteases (aureolysin/Aur), and serine protease-like proteins (spls) that directly destroy host tissue constituents, including collagen, elastin, and mucins [10]. *S. aureus* proteases degrade extracellular matrix components and immune proteins, thereby disrupting granulation tissue formation, diminishing collagen content, and compromising wound tensile strength, resulting in chronic, non-healing wounds [11]. *P. aeruginosa* utilizes the type III secretion system (T3SS) virulence factor to deliver effector toxins (ExoS, ExoT, ExoU, and ExoY), which contribute to tissue damage and impede wound healing [12]. Sepsis is a common and serious consequence in patients with diabetic ulcers, markedly exacerbating ulcer severity, elevating the risk of amputation, and raising mortality rates. Sepsis is a primary cause of mortality in patients with DFU, exhibiting mortality rates between 15% and over 30% within 5 to 10

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Table 1. Morphological characteristics of bacterial isolates from diabetic ulcers.

Bacterial isolate	Morphological characteristics			
	Color	Colony shape	Elevation	Margin
1PACP	cream with yellow-green pigments	circular	flat	entire
M91A	white colonies surrounded by a yellow zone	circular	raised	entire
E34P	blue white-centered	circular	raised	entire
E104U	purple	circular	raised	entire
EA4M	red	circular	raised	entire
P54A	cream with yellow-green pigments	irregular	flat	undulate
M74AP	white colonies surrounded by a yellow zone	circular	raised	entire
M34B	white colonies surrounded by a yellow zone	circular	raised	entire
M13B1	white colonies surrounded by a yellow zone	circular	raised	entire
M34KK	white colonies surrounded by a yellow zone	circular	raised	entire
E91B	purple dark-centered	circular	raised	entire
MA74AP	pink	circular	raised	entire
13MIPP	white colonies surrounded by a yellow zone	circular	raised	entire
14PIBC	cream	circular	raised	entire
P63AC2	cream	curled	raised	entire

years, particularly post-amputation [13].

Antibiotic resistance has been documented in bacteria derived from diabetic wounds, specifically *S. aureus* exhibiting resistance to benzylpenicillin, amoxicillin, dicloxacillin, oxacillin, ertapenem, imipenem, and tetracycline; *P. aeruginosa* resistant to ampicillin, cefazolin, ceftriaxone, ertapenem, tigecycline, nitrofurantoin, and trimethoprim; and *E. coli* resistant to ampicillin, cefazolin, ceftriaxone, aztreonam, and nitrofurantoin [14][15]. Biofilm formation further exacerbates resistance, shielding bacteria from host defences response through the biofilm matrix, creating a barrier to most antibiotic therapies [16]-[20]. The presence of multidrug-resistant organisms (MDRO) and biofilms in diabetic ulcer is strongly associated with increased risk of treatment failure and chronicity of wounds [21][22].

Identifying the blaTEM gene in bacteria isolated from diabetic foot ulcers is crucial for recognizing the synthesis of extended-spectrum beta-lactamase (ESBL) and informing appropriate antibiotic treatments. blaTEM encodes TEM-type beta-lactamases that confer resistance to numerous beta-lactam antibiotics, thereby complicating infection therapy. A significant prevalence of blaTEM was seen in bacteria isolated from diabetic foot ulcers, including *E. coli, Proteus mirabilis,* and

Acinetobacter baumannii [23][24]. Identifying the sul1 gene in diabetic ulcer infections is crucial for identifying sulfonamide and multidrug resistance, thereby informing successful antibiotic treatment. Sul1 gen is frequently identified in Gram-negative bacteria associated with diabetic ulcers [25][26].

Previous studies have recovered 70 bacterial strains with hemolytic capabilities from swabs of diabetic ulcers: 15 isolates were identified as β-hemolysins, eight as α-hemolysins, and 47 as γ-hemolysins. We identified common pathogens, such as *S. aureus* and *P. aeruginosa*, in the diabetic ulcer samples [27], but limited data exist on the molecular characterization of resistance genes in isolates from Indonesian patients. In particular, the prevalence of blaTEM and sul1 genes, associated with β-lactam and sulfonamide resistance respectively, remains underexplored. This study aims to characterize the bacterial diversity, antibiotic resistance profiles, and biofilm-forming capacity, with a focus on blaTEM and sul1 gene detection of isolates from diabetic ulcer patients in West Java, Indonesia. We hypothesize that these isolates exhibit multidrug resistance and harbor resistance genes detectable via PCR. To test this, we employed using the selective differential medium test identification, antibiotic susceptibility testing, biofilm assays, and molecular gene

detection.

2. MATERIALS AND METHODS

2.1. Sample Collection

Bacteria were isolated from diabetic ulcer swab samples of diabetic patients at the Balutan Luka Terkini '@nan' diabetic wound care clinic in South Tambun, West Java, Indonesia, beween May and June 2024. Media transport used 10 mL of phosphate-buffered saline. Ten patients, as a sample collection, who met the inclusion criteria, underwent one or more antimicrobial therapies (cefixime, azithromycin, clindamycin, ciprofloxacin, amoxicillin). In this study, diabetic ulcer swab samples were obtained from the foot area of ten diabetic patients. The isolated bacteria were grown on eosin methylene blue agar (EMBA, Oxoid, UK), maltose salt agar (MSA, Merck, Germany), and Pseudomonas cetrimide agar (Merck, Germany). The hemolytic activity test was performed using blood agar media. Antibiotic disks used ampicillin 10 µg/mL (Oxoid, UK), ciprofloxacin 5 µg/mL (Oxoid, UK), clindamycin 2 µg/mL (Oxoid, UK), erythromycin 15 µg/mL (Oxoid, UK), doxycycline 30 µg/mL (Oxoid, UK), and trimethoprim-sulfamethoxazole 25 µg/mL (Oxoid, UK). Genomic DNA from the bacterial isolates was extracted using the Presto^{TM} Mini gDNA Bacteria Kit (Geneaid, Taiwan). Ethical approval was obtained from the Human Research Ethics Committee of Bogor Agricultural University (1172/IT3.KEPMSM-IPB/SK/2024).

2.2. Isolation of the Diabetic Ulcer Swabs Sample Bacteria

Wound care patients with inclusion criteria had received one or more antibiotic therapies. Wound swab samples were collected by medical personnel using a sterile swab inserted into 10 mL phosphate buffered saline. The isolated bacteria were grown on EMBA, MSA, and Pseudomonas cetrimide agar medium.

Table 2. Antibiotic susceptibility profiles of bacterial isolates from diabetic ulcer swab samples. The MIC value of antimicrobials not evaluated for bacterial isolates is known as the NA.

Bacterial Isolate	Antibiotic susceptibility pattern					
	AM	CIP	CM	E	DO	SXT
1PACP	R	R	R	R	S	S
M91A	I	S	S	I	S	NA
E34P	S	S	R	I	S	NA
E104U	R	R	R	R	R	NA
EA4M	R	I	I	I	I	NA
P54A	R	S	R	R	S	NA
M74AP	R	S	S	I	S	NA
M34B	R	S	S	S	I	NA
M13B1	R	R	R	R	S	NA
M34KK	R	S	S	I	R	NA
E91B	S	I	S	S	S	NA
MA74AP	I	S	S	S	S	NA
13MIPP	I	R	R	R	S	S
14PIBC	R	R	R	R	R	R
P63AC2	R	R	R	R	R	NA

Note: *Standard of antibiotic concentration according to (CLSI Supplement M100, 2023). AM: Ampicillin; CIP: Ciprofloxacin; CM: Clindamicin; E: Erytrhomycin; DO: Doxycycline; SXT:Trimethoprim-Sulfamethoxazole, I: Intermediate, R: Resistant; S: Sensitive.
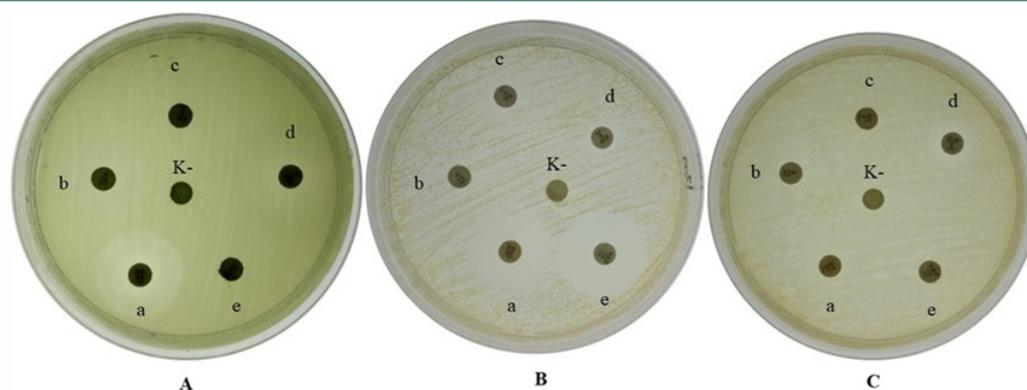
Figure 1. Zones of inhibition for different antibiotics after 24 h incubation at 37 °C. A. Isolate bacteria 1PACP, B. Isolate bacteria 13MIPP, C. Isolate bacteria 14PIBC, a. Doxycyclin, b. erytrhromycin, c. clindamycin, d. ciprofloxacin, e. ampicillin, K- : Negative control ddH$_2$O.

2.3. Antibiotic Susceptibility

Furthermore, the growing bacterial colonies were purified, subcultured, and tested for hemolytic activity and antibiotic resistance [28][29]. The hemolytic activity test was performed by scraping the bacterial culture on blood agar media. After 24 h of incubation at 37 °C, bacterial colonies were observed on blood agar media [28]. Tests for antibiotic resistance of bacteria isolated from diabetic ulcers were performed using antibiotic disks, six antibiotics (ampicillin 10 μg/mL, ciprofloxacin 5 μg/mL, clindamycin 2 μg/mL, erythromycin 15 μg/mL, doxycycline 30 μg/mL, and trimethoprim-sulfamethoxazole 25 μg/mL), and ddH$_2$O as a negative control. The six antibiotics originate from six distinct classes frequently employed in the treatment of Gram-positive and Gram-negative bacteria [28][29] and are additionally utilised for managing diabetic wound infections in patients from whom we obtained wound swab samples, specifically ciprofloxacin and clindamycin. The multiple antibiotic resistance (MAR) index was computed and analyzed in accordance with Krumperman method [30], utilizing the formula a/b, where 'a' denotes the quantity of antibiotics to which an isolate exhibited resistance, and 'b' signifies the total number of antibiotics evaluated [31]. In this study, the quantity of antibiotics (b) to which the isolate was subjected was 6. The assays were performed in duplicate.

2.4. Biofilm Formation Test Microtiter Plate Assay Method

A total of 100 μL of each bacterial suspension with an optical density value at a wavelength of 600 nm of 0.01 was put into the test wells in a sterile 96-well microplate. The negative control was 100 μL of nutrient broth (NB) medium (Merck, Germany). A 96-well microplate containing the bacterial suspension was incubated at 37 °C for 24 h used in an incubator excellent 256L INE600 (Memmert, Germany). After 24 h, the suspension of pathogenic bacteria on the microplate was discarded, and the microplate was rinsed three times with sterile distilled water and dried by inverting it onto absorbent tissue. Biofilm staining was performed by inserting 105 μL of 0.1% crystal violet solution (Merck, Germany) into each well and incubating on an incubator shaker at room temperature for 30 min used in an orbital shaking incubator model OSI-501D (Firstek Scientific, Japan). The microplate containing the crystal violet solution was then washed with distilled water and dried. Each well received 110 μL of 70% ethanol solution, which was allowed to incubate for 30 min at ambient temperature. Optical density (OD) was assessed to determine the turbidity of the solution using an Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, USA) at a wavelength of 595 nm and standardised to 0.2 [32]. The OD value obtained was then calculated as the value of OD c (cut-off value) and OD isolates to determine the category of pathogenic bacterial isolates as biofilm producers using the following Equations (1) and (2) [33].

$$\text{OD c} = \text{average negative control} + (3 \times \text{standard deviation of negative control}) \quad \textbf{(1)}$$

OD isolate = average OD isolate - OD c   (**2**)

Based on this, isolates were grouped into the following categories: strong biofilm producer (OD > 4 × ODc); moderate biofilm producer (4 × ODc ≥ OD > 2 × ODc); weak biofilm producer (2 × ODc ≥ OD > ODc); and non-biofilm producer (OD ≤ODc) [34]. According to the formula, the cutoff values were set as follows: Poor = OD595 < 0.18; Weak = 0.18 < OD595 < 0.37; Moderate = 0.37 < OD595 < 0.74; High = OD595 > 0.74 [35]. In this study, deviation standar negative control were 0.0229. All of the growth conditions were evaluated in triplicate.

2.5. Extraction of DNA

Genomic DNA was extracted using protocols outlined in the Presto^{TM} Mini gDNA Bacteria Kit (Geneaid, Taiwan). At first, 1×10^9 bacterial cells were transfered to a 1.5 mL microcentrifuge tube, centrifuge for 1 min at 14,000 × g used in an centrifuge K3 series (Centurion Scientific, UK), and discard the supernatant.

2.5.1. Gram-Positive Bacteria

Then, 200 µl of Gram-positive buffer was introduced into the tube, lysozyme (0.8 mg/200 µl) and the tube was vortexed until the lysozyme was fully dissolved.   Subsequently, a solution containing Gram (+) buffer supplemented with lysozyme was introduced into the sample, and the cell pellets were resuspended using a vortex or pipette and incubated at 37 °C for 30 min. The reverse was performed multiple times throughout the incubation period. Subsequently, 20 µL of proteinase K was added, vortexed to ensure thorough mixing, and incubated at 60 °C for 10 min used in an incubator excellent 256L INE600 (Memmert, Germany), and reverse every 3 min during incubation.

2.5.2. Gram-Negative Bacteria

Then, 180 µl of GT Buffer was added, and the cell pellets were resuspended using a pipette. Subsequently, 20 µL of proteinase K was added and vortexed to ensure thorough mixing. Incubate at 60 °C for 10 min used in an incubator, performing a reverse every 3 min during incubation. Subsequent process samples (Gram-positive and Gram-negative bacteria) were supplemented with 200 µL of GB Buffer, vortexed, and incubated for 10 min at 70 °C used in an incubator. Subsequently, 200 µL of 100% ethanol was added, followed by vortexing for 10 s, after which the mixture was transferred to a GD Column and centrifuged at 14,000 × g for 2 min. The collection tube was replaced with a new tube, 400 µl of W1 buffer was added, and the tube was centrifuged at 14,000 × g for 30 s. Subsequently, 600 µl of Wash Buffer was added and the mixture was centrifuged at 14,000 × g for
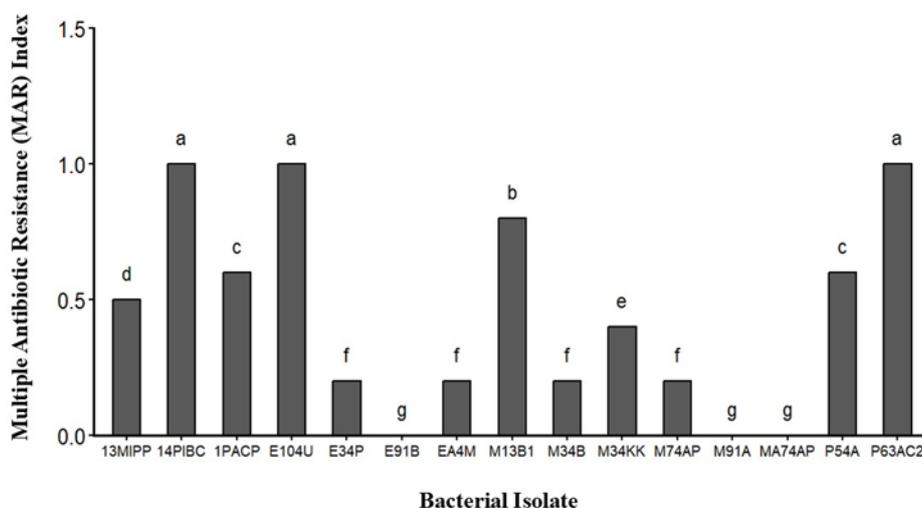

Figure 2. MAR index bacteria isolate from diabetic ulcer sample. Different letters above the bars in the figure indicate significantly different results based on Duncan's Multiple Range Test at a significance level of p < 0.05.
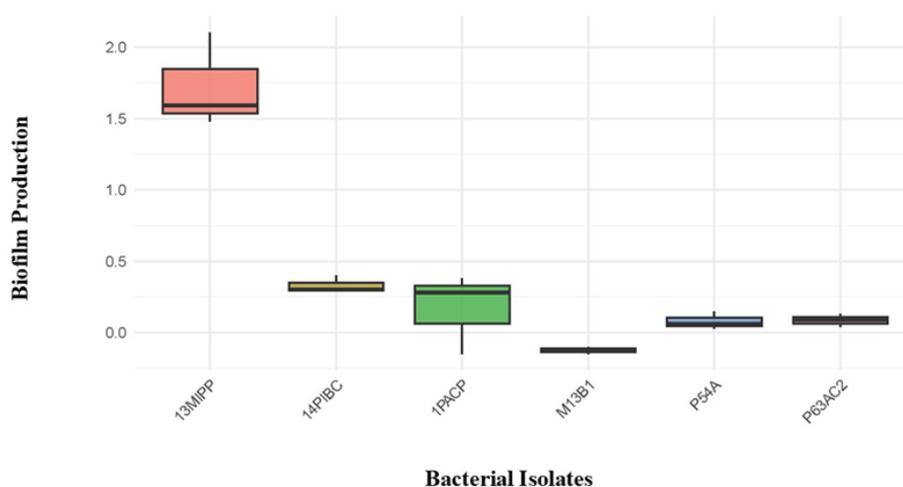
Figure 3. Biofilm formation of bacterial isolate using (tissue culture plate method by microplate reader). Bacterial isolate 13MIPP showed a box plot with the highest median value of approximately 1.7, compared to the other isolates, and was significantly different according to the results of Tukey's post hoc test, which showed a P-value of 0.000 when comparing 13MIPP with isolates 14PIBC, 1PACP, M13B1, P54A, and P63AC2.

30 s. The GD Column was then transferred to a sterile Eppendorf tube and 100 μl of preheated elution buffer was added. This mixture was then centrifuged at 14,000 × g for 30 s used in an centrifuge K3 series (Centurion Scientific, UK). The supernatant associated with the Eppendorf tube was a DNA extract [36].

2.6. Polymerase Chain Reaction (PCR) Amplifications

Three bacterial isolates with the best biofilm formation ability were selected for β-lactamase resistance gene detection, sulfonamide resistance gene detection, and identification using 16S rRNA-based methods. The detection of blaTEM and sul1 indicates antimicrobial resistance in patients who have been treated with class B-lactam antibiotics and sulfonamides [23][25]. The β-lactamase genes were amplified using the universal primers blaTEM Forward 5'-CATTTC CGTGTCGCCCTTATTC-3' and blaTEM Reverse 5'-CGTTCATCCATAGTTGCCTGAC-3'. The PCR protocol consisted of an initial denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 60 °C for 40 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 7 min using an Applied Biosystems 2720 thermal cycler (Applied Biosystems, USA). The amplification products were separated on a 0.8% agarose gel, using 100bp DNA ladders as molecular weight markers [37].

The sulfonamide genes were amplified using the universal primer sul1 Forward 5'-CGGCGTGGGCTACCTGAACG-3' and sul1 Reverse 5'-GCCGATCGCGTGAAGTTCCG-3'. The PCR protocol included an initial denaturation step at 94°C for 3 min, followed by denaturation at 94 °C for 40 s, 35 cycles of annealing at 55 °C for 40 s, elongation at 72 °C for 40 s, and a final post-elongation step at 72 °C for 2 min. The amplification products were resolved on a 0,8% agarose gel, utilizing 100 bp DNA ladders as molecular weight markers [38]. The 16s rRNA gene was amplified with the 1387r primer (5'-GGGCGGWGTGTACAAGGC-3') and 63f primer (5'-CAGGCCTAACACATGCAAGTC-3') [39], resulting in a 1,300 bp amplicon length. The amplification procedure was conducted over 35 cycles of pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, elongation at 72 °C for 1 min and 30 s, and post-elongation at 72 °C for 5 min. The amplicon was placed in a 1.5% agarose gel with a 1kb DNA ladders as molecular weight size markers.

2.7. Sequencing of PCR Products

DNA sequence analysis of the amplicons was conducted to identify the β-lactamase and

sulfonamide genes by PCR Applied Biosystems 2720 thermal cycler (Applied Biosystems, USA). Purified PCR products were subsequently sent for sequencing using Sanger sequencing at Apical Scientific Sdn. Bhd. in Selangor, utilizing the ABI PRISM® 377 DNA Sequencer from Applied Biosystems, USA. The acquired sequences were subsequently compared to those in the National Center for Biotechnology Information (NCBI) database using the Basic Alignment Search Tool (BLAST) [40]. Identification via 16S rRNA sequencing. The PCR product was sequenced at the First Base Saraswanti Genomics Institute in Indonesia. For sequence analysis, the raw sequence was edited based on the chromatogram quality. The shortened sequence was aligned using BLASTN against the NCBI database to identify the highest similarity. The phylogenetic tree was generated using Molecular Evolutionary Genetics Analysis (MEGA) X software employing the maximum parsimony method. The DNA sequences were submitted to GenBank at http://www.ncbi.nlm.nih.gov under the accession numbers: bacterial isolate 1PACP (PV052610), bacterial isolate 13MIPP (PV052608), bacterial isolate 14PIBC (PV052609), blaTEM gene; bacterial isolate 1PACP (PV394614), bacterial isolate 13MIPP (PV454647), bacterial isolate

14PIBC (PV454648), sul1 gene; bacterial isolate 13MIPP (PV613715), bacterial isolate 14PIBC (PV613716).

2.8. Statistical Analysis

Antibiotic susceptibility was descriptively analyzed. The MAR index was computed and analyzed in accordance with the Krumperman formula [30]. Graphs were generated using R Studio (Posit Software, PBC, USA) version 2025.09.0+387. Statistical analysis was performed using one-way ANOVA and Duncan's Multiple Range Test at a significance level of $p < 0.05$. Biofilm formation was quantified using the Stepanovic et al. formula [33]. Graphs were generated by plotting biofilm formation capacity versus bacterial isolates using R Studio (Posit Software, PBC, USA) version 2025.09.0+387. Groups were analyzed using one-way ANOVA and Tukey's post hoc test. Statistical significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSIONS

3.1. Bacterial Identification

Bacteria were isolated from diabetic ulcer swab samples of ten diabetic patients using three selective media: EMBA, MSA, and Pseudomonas
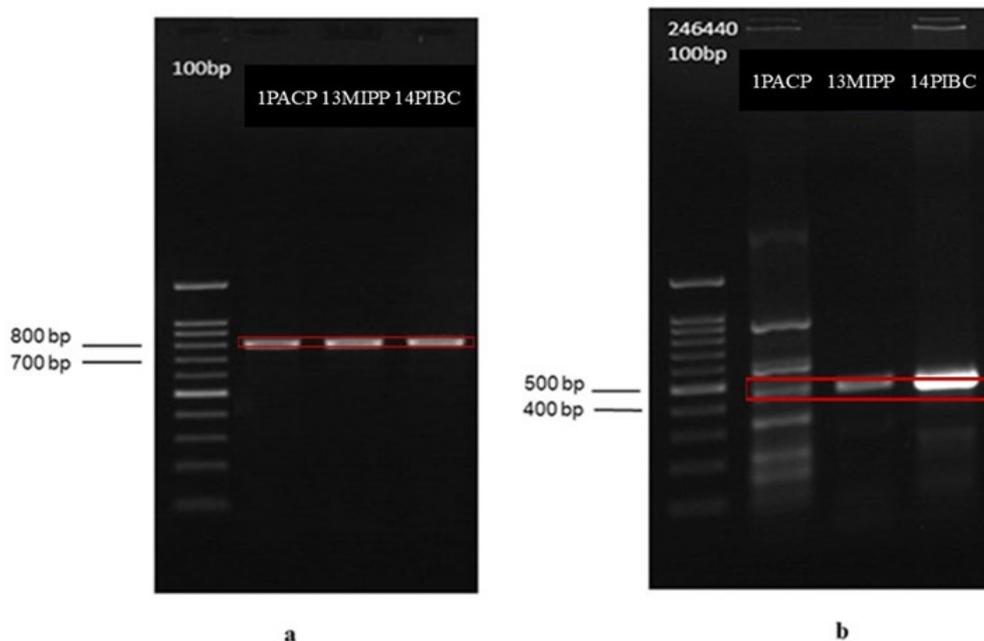

Figure 4. Visualization of blaTEM gene (a) and sul1 gene (b) amplicons of bacterial isolates code 1PACP, 13MIPP, and 14PIBC by agarose gel electrophoresis.

Table 3. Identities 1PACP, 13MIPP, and 14PIBC based on the blaTEM gene at ~800pb using BLAST-X.

Isolate	Description	Max score	Query cover (%)	E-value	Identity (%)	Sequence length	Accession no.
1PACP	TEM family class A beta-lactamase, partial [P. aeruginosa]	475	100	8e-168	100	738	WP-337156667.1
13MIPP	TEM family class A beta-lactamase [S. gallinarum]	486	100	4e-172	100	750	WP-119638253.1
14PIBC	TEM family class A beta-lactamase [P. aeruginosa]	479	99	2e-169	100	745	WP-337156667.1

Table 4. Identities 13MIPP and 14PIBC based on the sul1 gene at ~430pb using BLAST-X.

Isolate	Description	Max score	Query cover (%)	E-value	Identity (%)	Sequence length	Accession no.
13MIPP	Sulfonamide-resistant dihydropteroate synthase sul1, partial [E. coli]	253	99	9e-84	100	391	QUW44406.1
14PIBC	Dihydropteroate synthase, partial [P. aeruginosa]	234	86	1e-75	100	413	WP-083563989.1

cetrimide agar. Previous studies have recovered 70 bacterial strains with hemolytic capabilities from swabs of diabetic ulcers: 15 isolates were identified as β-hemolysins, eight as α-hemolysins, and 47 as γ-hemolysins [27]. Fifteen isolates identified as β-hemolysins in previous studies were tested for antibiotic susceptibility and their ability to form biofilms in this study. Fifteen bacterial isolate exhibited different morphological characteristics (Table 1). Bacterial isolate 13MIPP, obtained on MSA media, exhibited morphological traits including a circular form, raised elevation, entire margin, and white colonies surrounded by a yellow zone. Bacterial isolates were successfully obtained on Pseudomonas cetrimide agar medium, including bacterial isolate 1PACP, which exhibited morphological characteristics of a circular form, flat elevation, entire margin, and cream colony color with yellow-green pigments. Bacterial isolate 14PIBC displayed morphological traits of circular form, raised elevation, entire margin, and cream colony color (Table 1).

EMBA is a differential microbiological medium that provides a colorimetric distinction between lactose-fermenting organisms such as *E. coli* and non-fermenters. It partially restricts the proliferation of Gram-positive bacteria (e.g., *Staphylococci, Salmonella, Shigella*). Lactose is fermented by Gram-negative bacteria, resulting in a reduction in pH owing to acid production. Consequently, the colonies were more predisposed to dye assimilation. The reaction of the acid with the pigments caused the clusters to turn dark purple. Moreover, many lactose-fermenting bacteria generate flat black aggregates with green metallic shine [41].

Lowbury proposed the use of cetrimide (cetyltrimethylammonium bromide), which significantly suppresses the growth of associated microbial flora [42]. A concentration of 0.3 g/L effectively suppressed the surrounding organisms and reduced interference with the growth of *P. aeruginosa*. Pigment synthesis by *P. aeruginosa* was not suppressed when cultivated in this medium. *P. aeruginosa* in a medium containing yellow-green pigments. Mannitol salt agar LI in 90 mm settling plates is a modified variant of the selective agar introduced by Chapman [43] for isolating and presumptively identifying *S. aureus*. The formulation of mannitol salt agar facilitates the

proliferation of *S. aureus*, whereas an elevated salt concentration of 7.5% inhibits numerous other bacteria. Unlike most other staphylococci, *S. aureus* metabolizes mannitol to produce acids. Consequently, they formed yellow colonies accompanied by yellow zones. The potential presence of *S. aureus* is indicated by the proliferation of yellow or white colonies encircled by a yellow halo. Based on the morphological characteristics in Table 1, bacterial isolates 1PACP and 14PIBC exhibit morphological similarities to those of Gram-negative *Pseudomonas*. Bacterial isolates 13MIPP, M91A, M74AP, M34B, M13B1, M34KK, and share morphological characteristics similar to those of Gram-positive *Staphylococcus*. To determine the species of the 15 isolates in Table 1, molecular identification of the 16S rRNA gene is required. In Southeast Asian diabetic foot ulcers, particularly in Bangladesh, Malaysia, and Indonesia, Gram-negative bacteria are the predominant isolated pathogens, frequently surpassing Gram-positive bacteria in prevalence [44]-[47]. The predominant isolates comprise *Klebsiella pneumoniae, Proteus* spp., *Pseudomonas aeruginosa, Escherichia coli*, and *Staphylococcus aureus* [44][46].

3.2. Antibiotic Susceptibility

The results of the antibiotic clear zone against bacteria that caused infection in diabetic ulcer samples (Table 2) showed that some bacterial isolates were resistant to one to six antibiotics tested. Multidrug-resistant (MDR) strains exhibit resistance to at least one antimicrobial across more than three classes of antimicrobials; an extensively drug-resistant (XDR) strain demonstrates resistance to at least one antimicrobial in all classes of antimicrobials, whereas a pan-drug-resistant (PDR) strain is resistant to all antimicrobial agents [48].

The specific bacterial isolates exhibited varying levels of antibiotic resistance (Table 2). **Ampicillin:** - Resistant isolates: 1PACP, E104U, EA4M, P54A, M74AP, M34B, M13B1, M34KK, 14PIBC, P63AC2 - Intermediate isolates: M91A, MA74AP, 13MIPP - Sensitive isolates: E34P, E91B. **Ciprofloxacin:** - Resistant isolates: 1PACP, E104U, M13B1, 13MIPP, 14PIBC, P63AC2 - Intermediate isolates: EA4M, E91B - Sensitive isolates: M91A, E34P, P54A, M74AP, M34B, M34KK, MA74AP. **Clindamycin:** - Resistant isolates: 1PACP, E34P, E104U, P54A, M13B1, 13MIPP, 14PIBC, P63AC2 - Intermediate isolate: EA4M - Sensitive isolates: M91A, M74AP, M34B, M34KK, E91B, MA74AP. **Erythromycin:** - Resistant isolates: 1PACP, E104U, P54A, M13B1, 13MIPP, 14PIBC, P63AC2 - Intermediate isolates: M91A, E34P, EA4M, M74AP, M34KK - Sensitive isolates: M34B, E91B, MA74AP. **Doxycycline:** - Resistant isolates: E104U, M34KK, 14PIBC,
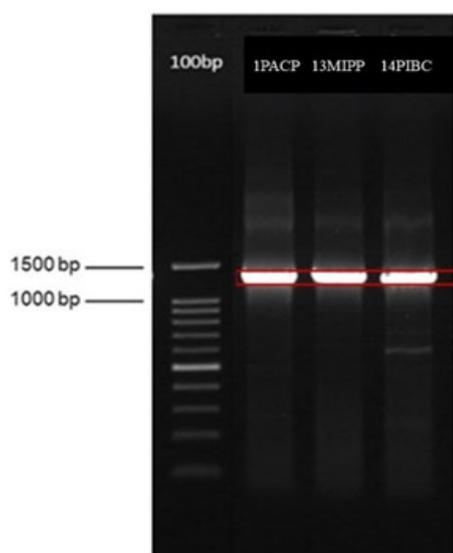

Figure 4. Visualization of blaTEM gene (a) and sul1 gene (b) amplicons of bacterial isolates code 1PACP, 13MIPP, and 14PIBC by agarose gel electrophoresis.

P63AC2 - Intermediate isolates: EA4M, M34B - Sensitive isolates: 1PACP, M91A, E34P, P54A, M74AP, M13B1, E91B, MA74AP, 13MIPP. **Trimethoprim-Sulfamethoxazole:** - Resistant isolate: 14PIBC - Sensitive isolates: 1PACP, 13MIPP. Thus, this overview summarizes the resistance patterns of the bacterial isolates to the specified antibiotics.

Bacterial isolate 1PACP was resistant to the antibiotics ampicillin, ciprofloxacin, clindamycin, and erythromycin, but was still sensitive to doxycycline. Bacterial isolate 13MIPP was resistant to ciprofloxacin, clindamycin, and erythromycin, had intermediate resistance to ampicillin, and remained sensitive to doxycycline. Isolate 14PIBC was resistant to ampicillin, ciprofloxacin, clindamycin, erythromycin, and doxycycline (Figure 1). Ampicillin belongs to the beta-lactam class, and Ciprofloxacin belongs to the quinolone class, specifically the fluoroquinolones. Doxycycline belongs to the tetracycline class, and clindamycin belongs to the lincosamide class. Trimethoprim-Sulfamethoxazole belongs to the sulfonamide class.

Bacterial isolates 1PACP, E104U, M13B1, 13MIPP, 14PIBC, and P63AC2 were resistant to the antibiotic ciprofloxacin, which belongs to the quinolone class, specifically fluoroquinolones. Other studies have also reported similar findings, gram-negative bacteria (notably *E. coli, Klebsiella pneumoniae*, and *P. aeruginosa*) exhibiting high rates of MDR and resistance to cephalosporins and fluoroquinolones [25][49]. Methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus* are also frequently reported among gram-positive isolates [25][49][50]. The emergence of antibiotic-resistant bacteria in these wounds complicates therapy, restricts therapeutic alternatives, and deteriorates patient outcomes. Empirical antibiotic therapy has been increasingly challenged by resistance, necessitating regular local surveillance and targeted treatment based on the culture and sensitivity results [25][49].

3.3. MAR Index Analysis

The MAR index is a widely used metric to quantify the extent of antibiotic resistance in bacterial isolates from clinical settings. Bacterial isolate E104U is resistant to 5 types of antibiotics

Table 5. BLASTN results against the NCBI database for the trimmed nucleotide sequence of partial 16S rRNA.

Isolate	Description	Max score	Query cover (%)	E-value	Identity (%)	Sequence length	Accession no.
1PACP	*P. aeruginosa* strain DSM 50071	2244	100	0.0	98,74	1268	NR_117678.1
13MIPP	*S. aureus* strain ATCC 12600	2346	100	0.0	96.84	1276	NR_115606.1
14PIBC	*P. aeruginosa* strain DSM 50071	2037	94	0.0	97.88	1256	NR_117678.1

against five antibiotics tested and has a MAR index of 1. Bacterial isolate P63AC2 is resistant to 5 types of antibiotics against five antibiotics tested and has a MAR index of 1. Bacterial isolate 14PIBC is resistant to 6 types of antibiotics against six antibiotics tested and has a MAR index of 1. Bacterial isolate M13B1 was resistant to four types of antibiotics against the five antibiotics tested and had a MAR index of 0.8. Bacterial isolate 1PACP is resistant to four types of antibiotics against the six antibiotics tested and has a MAR index of 0.6. Bacterial isolate 13MIPP was resistant to three types of antibiotics out of the six tested and had a MAR index of 0.5. The M34KK bacterial isolate was resistant to 2 types of antibiotics out of the five antibiotics tested and had a MAR index of 0.4. The E34P, EA4M, M74AP, and M34B bacterial isolates were each resistant to one type of antibiotic out of the five antibiotics tested and had a MAR index of 0.2. Meanwhile, bacterial isolates M91A, E91B, and MA74AP had a MAR index of 0. MAR index bacteria isolated from a diabetic ulcer sample are shown in Figure 2. Bacterial isolates with an MAR index $\geq$ 0.4 will be tested for their ability to form biofilms. Bacterial isolates with MAR index $\geq$ 0.4 were bacterial isolates 1PACP, M13B1, 13MIPP, P54A, 14PIBC, and P63AC2.

Figure 2 shows the MAR index values of the 15 bacterial isolates. The highest MAR index values were observed in bacterial isolates 14PIBC, E104AU, and P63AC2, which showed no significant differences in MAR index values. Meanwhile, the bacterial isolates 14PIBC, E104AU, and P63AC2 were significantly different from the bacterial isolates M13B1, 1PACP, P54A, 13MIPP, M34KK, E34P, EA4M, M34B, M74AP, E91B, M91A, and MA74AP. Bacteria with an MAR score of > 0.2 come from a high-risk source of contamination that uses multiple antibiotics [51]. MAR index 0.4–1.0 indicates high resistance [52]. Bacterial isolates with MAR index $\geq$ 0.4 were bacterial isolates 1PACP, M13B1, 13MIPP, P54A, 14PIBC, and P63AC2. The six bacterial isolates were tested for their ability to form biofilms.

3.3. Biofilm Formation

Figure 3 shows that five bacterial isolates formed biofilms, and one bacterial isolate did not form a biofilm (bacterial isolate M13B1). Based on their ability to form biofilms, five bacterial isolates were classified as poor (bacterial isolates P54A and P63AC2), weak (bacterial isolates 1PACP and 14PIBC), and moderate (bacterial isolate code 13MIPP). Bacterial isolates with weak or moderate biofilm formation ability and an MAR index $\geq$ 0.4 will be subjected to DNA isolation, β-lactamase, and sulfonamide genes detected by PCR and sequencing.

Bacterial isolate 13MIPP revealed that a Box Plot with a median line of approximately 1.7. This indicated that the median biofilm production in isolate 13MIPP was the highest among all other isolates and was significantly different from that of the other isolates. This is consistent with the results of Tukey's post hoc test, which showed a P-value of 0.000 when comparing 13MIPP with samples 14PIBC, 1PACP, M13B1, P54A, and P63AC2. The bacterial isolate M13B1 showed the lowest median (close to zero or negative), indicating no biofilm production. The bacterial isolates P54A, 14PIBC, P63AC2, and 1PACP had medians at similar and overlapping levels. This is consistent with Tukey's post hoc test, which showed no significant difference (p-value greater than 0.05) when comparing each bacterial isolate: P54A, 14PIBC, P63AC2, and 1PACP.

Quantitative analysis of biofilm formation was conducted using crystal violet staining of bacterial isolates from diabetic ulcers (Figure 3). Biofilms demonstrate a greater tolerance to antibiotics owing to various mechanisms. Extracellular polymeric substance (EPS) matrix inhibits the penetration of antibiotics. Additionally, gradients of nutrients and oxygen create areas of low metabolic activity, contributing to antibiotic tolerance. Bacteria within biofilms also have high mutation rates and can express efflux pumps and other resistance mechanisms. Furthermore, the bacteria in biofilms communicate with one another to regulate gene expression, thereby enhancing their resistance to antibiotics [16].

High rates of MDR have been observed among biofilm-forming bacteria, especially *Staphylococcus aureus, Pseudomonas aeruginosa*, and gram-negative bacilli [53]. Biofilm production significantly contributes to the chronicity of DFU, resulting in prolonged wound healing and an elevated risk of amputation [54]. Antibiotic
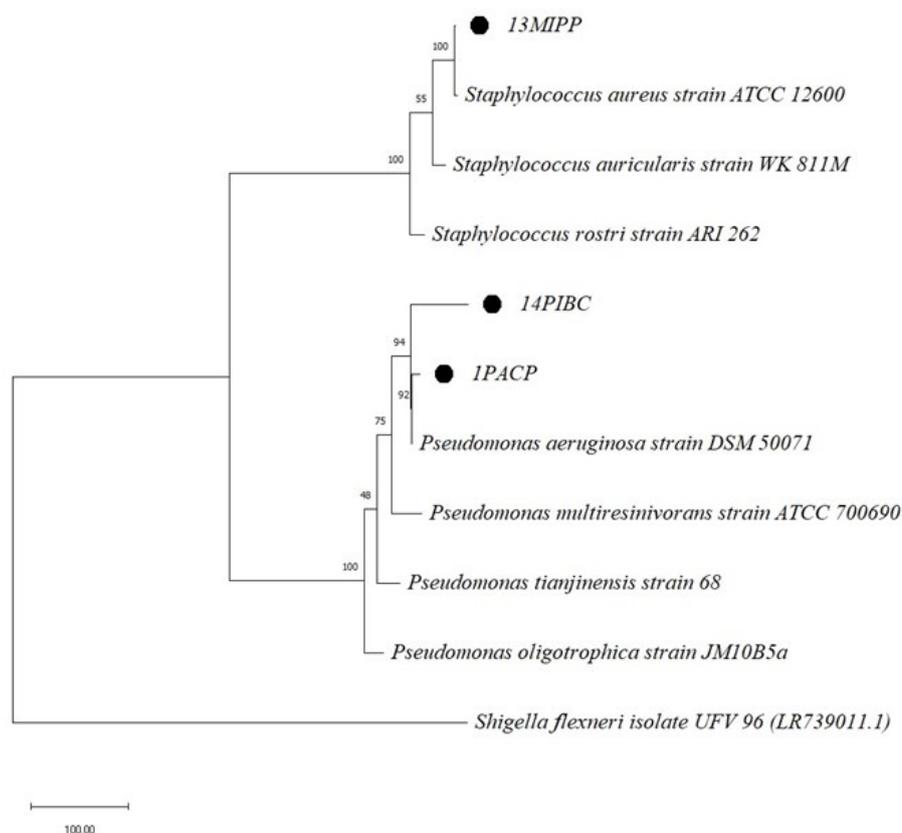
Figure 6. A phylogenetic tree depicting partial 16S rRNA sequencing of the bacterial isolates coded as 1PACP, 13MIPP, and 14PIBC. The tree was constructed using the maximum parsimony method with MEGAX software.

efficiency is diminished by the protective matrix and modified bacterial metabolism [21][55]. Clinical identification is challenging because the presence of biofilms does not consistently correspond with observable indications of infection [56].

3.3. PCR Detection of 16S rRNA and Resistance Gen

PCR product bands of blaTEM amplification of isolates 1PACP, 13MIPP, and 14PIBC. The product was approximately 800 bp in size. Amplified blaTEM genes of isolates 1PACP, 13MIPP, and 14PIBC by agarose gel electrophoresis are shown in Figure 4(a). PCR product bands of sul1 amplification of isolates 1PACP, 13MIPP, and 14PIBC. The product was approximately 432 bp in size. Amplified sul1 genes of isolates 1PACP, 13MIPP, and 14PIBC by agarose gel electrophoresis are shown in Figure 4(b).

Identities 1PACP, 13MIPP, and 14PIBC based on the blaTEM gene at ~800pb using BLAST-X are shown in Table 3. Identities 13MIPP and 14PIBC based on the sul1 gene at ~430pb using BLAST-X are shown in Table 4. Band of PCR products from amplification of 16S rRNA in isolates are shown in Figure 5.

BLASTN results against the NCBI database for the trimmed nucleotide sequence of partial 16S rRNA are shown in Table 5. A phylogenetic tree depicting the partial 16S rRNA sequences of the bacterial isolates 1PACP, 14PIBC and 13MIPP are shown in Figure 6. Bacterial isolates coded as 1PACP (see Table 3) were closely related to the TEM family class A beta-lactamase, partial from *Pseudomonas aeruginosa* (with 100% identity). Similarly, isolate 13MIPP showed a strong relationship with the TEM family class A beta-lactamase from *Staphylococcus gallinarum* (also with 100% identity). Furthermore, isolate 14PIBC was closely related to TEM family class A beta-lactamase from *Pseudomonas aeruginosa* (100% identity). This information aligns with findings related to diabetic ulcers, where the infections were

attributed to *S. aureus* (36.90%) and *P. aeruginosa* (21.30%). Notably, 26 isolates of *P. aeruginosa* demonstrated resistance to several antibiotics: 33.33% were resistant to ciprofloxacin, 46.67% to clindamycin, 100% to ampicillin, and 100% to trimethoprim/sulfamethoxazole. In contrast, 45 isolates of *S. aureus* exhibited the following resistance rates: ampicillin (4.55%), clindamycin (20.45%), ciprofloxacin (43.18%), and 63.64% to trimethoprim/sulfamethoxazole. Additionally, detection of the BlaTEM gene (with a product size of 518 bp) indicated that extended-spectrum beta-lactamases (ESBLs) were predominantly found in isolates of *E. coli*. The prevalence of ESBL genes in *E. coli* is largely attributed to the blaTEM variant [57]. Identifying beta-lactamase genes in isolates from diabetic ulcers is essential for understanding antibiotic resistance patterns and for enhancing treatment strategies for infections associated with diabetic ulcers. CTX-M, a beta-lactamase gene, was found in 58.8% of *P. aeruginosa* isolates from DFU patients, indicating the significant presence of this resistance factor [58].

Carbapenemases, enzymes that confer resistance to carbapenem antibiotics, are classified into three principal Ambler classes: A, B, and D, according to their molecular structure and catalytic function. Classes A and D are serine β-lactamases, whereas class B includes metallo-β-lactamases that require zinc. Class A and D (serine β-lactamases) utilize a serine residue at the active site to hydrolyze β-lactam antibiotics, including [59]. Class A includes enzymes such as KPC, SME, IMI, NMC, and GES, and Class D includes OXA-type carbapenemases. Class B (metallo-β-lactamases) necessitate one or two zinc ions at the active site for functionality and do not utilize a serine residue. Class B encompasses IMP, VIM, NDM, and associated enzymes [60]. Zinc ions catalyze the hydrolysis of a water molecule that directly attacks the β-lactam ring [61].

The identification of VEB and GES genes, classified as class A lactamases, linked to extended-spectrum beta-lactamase (ESBL) formation in diverse gram-negative bacteria from DFU, has been established. The VEB gene was identified in *Proteus* spp., *Morganella morganii, Klebsiella oxytoca*, and *Acinetobacter baumannii*. In contrast, the GES gene has been observed in *E. coli, Proteus* spp., *Morganella morganii*, and *A. baumannii*. These genes confer resistance to contemporary β-lactams, thus complicating therapeutic alternatives [62]. The presence of β-lactamase genes such as CTX-M, TEM, SHV, OXA-1, VEB, and GES in DFU isolates underscores the challenge of treating infections with standard antibiotics. The high prevalence of these genes suggests a need for continuous monitoring of antibiotic resistance patterns and the development of targeted treatment strategies to manage infections effectively [58][62].

Detecting various β-lactamase genes in bacterial isolates from diabetic ulcers highlights the complexity of antibiotic resistance in these infections. Understanding the distribution and prevalence of these genes is essential for developing effective treatment protocols and mitigating the effects of resistant bacterial strains. Bacterial isolates code 13MIPP (see Table 4) were found to be closely related to the Sulfonamide-resistant dihydropteroate synthase Sul1, partial from *Escherichia coli* (with identity 100%). Furthermore, isolate 14PIBC were closely related to Dihydropteroate synthase, partial from *Pseudomonas aeruginosa* (with identity 100%).

The second category of sulfa resistance is linked to genes (sul), usually located on plasmids present in clinical isolates of gram-negative organisms, such as *E. coli, A. baumannii*, and *K. pneumoniae* [63][64]. Plasmid-mediated sulfonamide resistance was initially recorded in the 1950s and the 1960s in *Shigella* and *Escherichia coli*. Subsequently, four primary mobilizable sul genes, sul1, sul2, sul3, and sul4, have been identified, each facilitating the global dissemination of sulfonamide resistance across various bacterial populations. sul1 was initially detected in 1975 in *E. coli* and *Citrobacter* sp., sul2 in 1980 in UTI-causing *E. coli*, sul3 in 2003 in *E. coli* from pigs, and sul4 in an unnamed bacterium discovered in wastewater in 2017 [65]. The CARD Database indicated that 40% of *P. aeruginosa*, 16% of *Enterobacter cloaceae*, 18% of *K. pneumoniae*, and 44% of *A. baumannii* genomes possess sul1, demonstrating its extensive dissemination [66].

Venkatesan et al. [65] reported that Sul enzymes possess a restructured pABA-binding domain and exhibit distinct conformational dynamics in their active regions, relative to DHPS. Research

indicates that Sul enzymes include an extra phenylalanine residue absent in DHPS, obstructing sulfonamide binding, hence serving as the critical molecular component in Sul-mediated sulfonamide resistance. The study indicated that Sul enzymes can proficiently substitute the function of DHPS in folate production in *E. coli*. The result also unequivocally confirms the ineffectiveness of sulfa medicines like SMX, non-inhibiting Sul enzymes, despite their dihydropteroate synthase enzymatic characteristics like those of DHPS enzymes. Enzymatic research has indicated that this disparity is attributable to the capacity of Sul enzymes to differentiate between pABA and SMX molecules [65].

Bacteria designated as 1PACP, 13MIPP, and 14PIBC were effectively amplified, yielding PCR products of approximately 1400 bp (Figure 5). Isolate bacteria coded as 1PACP were closely related to *P. aeruginosa* strain DSM 50071, with 98.74% identity of 1268 bp aligned. Isolate bacteria coded as 13MIPP were closely related to *S. aureus* strain ATCC, with 96.84% identity of 1276 bp aligned. Isolate bacteria coded as 14PIBC were closely related to *P. aeruginosa* strain DSM 50071, with 97.88% identity of 1256 bp aligned (Table 5). The phylogenetic tree based on nucleotide sequences revealed that the isolated bacteria coded as 1PACP and 14PIBC (Figure 6) were closely related to the *P. aeruginosa* strain DSM 50071 reference strain. Isolate bacteria coded as 13MIPP (Figure 6) were closely related to *S. aureus* strain ATCC 12600. *S. aureus* and *P. aeruginosa* bacteria have been extensively documented as pathogens in diabetic ulcers [6]-[9]. This study contributes to the growing body of evidence on multidrug resistance in chronic wounds and highlights the need for molecular surveillance of resistance genes in clinical microbiology settings. Limitations of this study include the relatively small sample size and focus on only two resistance genes. Future research should explore broader genomic profiling and assess the clinical outcomes associated with resistant infections in diabetic ulcers.

4. CONCLUSIONS

This study identified 15 antibiotic-resistant bacterial isolates. Five isolates formed biofilms. Two isolates were weak biofilm formers, one was a moderate biofilm former, and two were poor biofilm formers. Bacterial isolates with MAR index $\geq$ 0.4 were bacterial isolates 1PACP, M13B1, 13MIPP, P54A, 14PIBC, and P63AC2. Bacterial isolate 13MIPP forming moderate biofilm, 1PACP and 14PIBC forming weak biofilm. Three bacterial isolates (1PACP, 13MIPP, 14PIBC) also had blaTEM and sul1 resistance genes. This study is similar to previous reports on the high rates of MDR observed among biofilm-forming bacteria, especially *S. aureus* and *P. aeruginosa*. In previous reports, blaTEM was seen in bacteria isolated from diabetic foot ulcers, including *E. coli, Proteus mirabilis*, and *Acinetobacter baumannii*, and Sul1 was frequently identified in gram-negative bacteria. In this study, *S. aureus* and *P. aeruginosa* were successfully identified as having blaTEM and sul1 resistance genes. These findings extend the existing knowledge. The implication of this study is the treatment of diabetic ulcer infections by determining the appropriate and effective type of antibiotics. Limitations of this study include the relatively small sample size and focus on only two resistance genes. Future research should explore broader genomic profiling and assess the clinical outcomes associated with resistant infections in diabetic ulcers.

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Conflicts of Interest

The authors declare no conflict of interest.

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DECLARATION OF GENERATIVE AI

Not applicable.

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