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Molecular Characterization and Biofilm Dynamics of Multi-Drug-Resistant Bacterial Isolates from Kenyan Diabetic Foot Ulcers

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Abstract

Diabetic foot ulcers (DFUs) are a major complication of diabetes mellitus and are frequently complicated by infections caused by multidrug-resistant (MDR) bacteria, resulting in prolonged hospitalization, poor wound healing, and increased risk of lower-limb amputation. This study investigated the molecular resistance determinants, virulence characteristics, and antibiofilm activity of MDR bacterial isolates recovered from infected DFUs in Kisumu County, Kenya. A cross-sectional study was conducted between March and July 2025 among 471 patients attending three referral hospitals. Following bacterial isolation and antimicrobial susceptibility testing, nineteen MDR isolates were selected for phenotypic and molecular characterization. Virulence factor production, antibiofilm activity, and detection of mecA, blaCTX-M-1, blaSHV, blaTEM, and blaKPC genes were performed using standard microbiological and PCR-based methods. Five of nine (55.6%) Gram-positive MDR isolates produced at least one virulence factor, while five of ten (50.0%) Gram-negative isolates produced all virulence factors evaluated. All seven-virulence factor-producing isolates retained biofilm-forming ability despite partial inhibition by selected antibiotics. The mecA gene was detected in 55.6% *Staphylococcus aureus* isolates, whereas blaCTX-M-1 was identified in 60% Gram-negative isolates. One *Acinetobacter baumannii* isolate co-harbored blaCTX-M-1 and blaSHV, while blaTEM and bla < sub>KPC</sub> were not detected. These findings demonstrate the circulation of MDR pathogens carrying clinically important resistance determinants and virulence traits among patients with infected DFUs in western Kenya, underscoring the need for strengthened molecular surveillance, antimicrobial stewardship, and evidence-based management strategies.

Introduction

Diabetic foot ulcers (DFUs) are among the most serious complications of diabetes mellitus and represent a major cause of morbidity, disability, and healthcare expenditure worldwide. Approximately 19–34% of individuals with diabetes are estimated to develop a DFU during their lifetime, and up to 60% of these ulcers become infected, substantially increasing the risks of prolonged hospitalization, osteomyelitis, lower-extremity amputation, and mortality [1]. Current clinical guidelines emphasize early diagnosis of infection and prompt initiation of antimicrobial therapy guided by microbiological culture, antimicrobial susceptibility testing, and clinical response to improve patient outcomes [2].

The development of DFUs is multifactorial, resulting from peripheral neuropathy, peripheral arterial disease, impaired immunity, and delayed wound healing associated with chronic hyperglycaemia [3]. These pathological changes create a favourable environment for microbial colonization and infection, frequently resulting in polymicrobial wounds containing both aerobic and anaerobic bacteria. Among the predominant aerobic pathogens are members of the ESKAPE group, including *Enterococcus* spp., *Staphylococcus* spp., *Klebsiella* spp., *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., which are recognized for their ability to evade antimicrobial therapy and contribute to chronic wound persistence [4]. The increasing prevalence of multidrug-resistant (MDR) pathogens in DFUs has further complicated clinical management by reducing treatment options and increasing the risk of treatment failure [5].

Multidrug-resistant pathogens possess numerous mechanisms that promote persistence within chronic wounds. Besides antimicrobial resistance, many of these organisms' express virulence factors, including extracellular enzymes and toxins, and readily form biofilms that protect bacterial communities from host immune responses and antimicrobial agents. Biofilm-associated infections are particularly difficult to eradicate and are associated with delayed wound healing, recurrent infection, and increased antimicrobial tolerance [6]. Consequently, understanding both the virulence characteristics and biofilm-forming capacity of MDR pathogens is essential for developing more effective treatment strategies for infected DFUs.

Among MDR pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) remains one of the most clinically important Gram-positive organisms associated with DFUs. Methicillin resistance is primarily mediated by the mecA gene, which encodes the altered penicillin-binding protein PBP2a and confers resistance to nearly all β-lactam antibiotics [7]. Similarly, Gram-negative pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* frequently produce extended-spectrum β-lactamases (ESBLs) and carbapenemases, including enzymes encoded by blaCTX-M, blaSHV, blaTEM, and blaKPC, which hydrolyse β-lactam antibiotics and contribute to resistance against multiple antimicrobial classes [8]. The dissemination of these resistance determinants, together with enhanced virulence and biofilm formation, presents a major challenge to the effective management of diabetic foot infections.

Although antimicrobial resistance among wound pathogens has been increasingly reported in East Africa, molecular epidemiological data describing MDR pathogens associated with DFUs remain limited in Kenya, particularly in Kisumu County. A previous study in Kisumu detected the mecA gene among MRSA isolates recovered from wound infections [9]. Elsewhere, the prevalence of mecA among MRSA isolated from DFUs ranges from 40% to 100% in studies conducted in Tunisia and India [10, 11]. Likewise, ESBL-associated genes have been widely reported in Gram-negative pathogens causing DFUs, including bla < sub>SHV</sub> (84.6%), blaTEM (75%), and blaCTX-M (76.9%) in Pakistan [12], and blaCTX-M(34.2%) and blaTEM (89.4%) in northern India [13]. In Uganda, mecA prevalence of 29% and 46% has been reported among MRSA isolates from eastern and western regions, respectively [14, 15], while bla < sub>CTX-M</sub>, blaSHV, and blaTEM have also been detected at high frequencies in MDR Gram-negative bacteria [16]. Similarly, high prevalence of blaCTX-M has been reported among clinical isolates from Mulago National Referral Hospital in Uganda and Kisii County Referral Hospital in Kenya [17, 18]. These findings demonstrate considerable geographical variation in antimicrobial resistance profiles and emphasize the importance of localized molecular surveillance to inform empirical antimicrobial therapy and antimicrobial stewardship.

Despite the growing burden of MDR infections in diabetic foot ulcers, limited information is available regarding the molecular resistance mechanisms, virulence characteristics, and biofilm-forming potential of bacterial pathogens causing DFUs in western Kenya. Addressing this knowledge gap is essential for improving patient management and informing infection prevention strategies. Therefore, this study aimed to characterize antimicrobial resistance genes, virulence factors, and the antibiofilm activity of selected antibiotics among multidrug-resistant bacterial isolates recovered from patients with infected diabetic foot ulcers in Kisumu County, Kenya.

Methodology

Study design and setting

This study employed a hospital-based cross-sectional design conducted between March and July 2025 in Kisumu County, Kenya. The study sites included three high-volume referral facilities: Jaramogi Oginga Odinga Teaching and Referral Hospital, Kisumu County Hospital, and Lumumba County Hospital, all of which are located along the Kisumu to Kakamega road. These facilities serve a large population of patients with diabetes mellitus and associated complications, including diabetic foot ulcers (DFUs).

Study population and sampling

The study population comprised patients with diabetes mellitus presenting with clinically infected diabetic foot ulcers (DFUs) at the three selected hospitals during the study period. A total of 471 participants were enrolled using a stratified random sampling approach combined with multistage sampling to ensure adequate representation across the participating health facilities. Eligible participants were adults with a confirmed diagnosis of diabetes mellitus who were presented with clinically infected DFUs, provided written informed consent, and had not initiated antibiotic therapy before specimen collection. Patients who declined to participate or had already commenced antibiotic treatment were excluded from the study. Wound specimens collected from all enrolled participants underwent bacteriological culture, bacterial identification, and antimicrobial susceptibility testing. Of the isolates recovered, 19 multidrug-resistant (MDR) isolates exhibited resistance to the antimicrobial agents tested and were subsequently selected for further characterization. These MDR isolates were evaluated for virulence factor production, biofilm-forming ability, and genes to characterize the genetic determinants of antimicrobial resistance, virulence, and other clinically relevant genomic features.

Isolation and identification of bacterial pathogens

Clinical specimens were aseptically collected from infected diabetic foot ulcers (DFUs) using sterile swabs and processed according to standard microbiological procedures as previously described [9]. Specimens were cultured on appropriate selective and differential media, and bacterial isolates were identified using conventional colony morphology, Gram staining, and standard biochemical tests.

Antimicrobial susceptibility testing (AST) was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar following the Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. Antibiotic selection was based on CLSI recommendations and local prescribing practices for the management of diabetic foot infections and included representatives of the major antimicrobial classes commonly used in clinical settings. Zone diameters were interpreted according to the current CLSI breakpoints.

Methicillin-resistant *Staphylococcus aureus* (MRSA) was identified phenotypically using the cefoxitin (30 µg) disk diffusion test, with isolates interpreted according to CLSI criteria. Extended-spectrum β-lactamase (ESBL) production among Gram-negative isolates was determined phenotypically using the combination disk method with cefotaxime and ceftazidime alone and in combination with clavulanic acid, in accordance with CLSI recommendations. An increase of ≥ 5 mm in the inhibition zone diameter for either antimicrobial agent in the presence of clavulanic acid compared with the antimicrobial alone was considered indicative of ESBL production.

Selection of multidrug-resistant isolates

Multidrug resistance (MDR) was defined according to the international consensus criteria as acquired non-susceptibility to at least one antimicrobial agent in three or more antimicrobial classes. In the present study, isolates exhibiting resistance to at least one agent in three or more of the antimicrobial classes tested were classified as MDR.

Following antimicrobial susceptibility testing, bacterial isolates recovered from the 471 study participants were screened for MDR. Nineteen isolates fulfilled the MDR definition and were therefore included in subsequent phenotypic and molecular analyses. These isolates comprised clinically important pathogens associated with diabetic foot infections, including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Prior to downstream analyses, all MDR isolates were subcultured on Mueller–Hinton agar to confirm purity and viability.

Detection of virulence factors

Virulence phenotypes of the MDR isolates (*A. baumannii, E. coli, S. aureus, K. pneumoniae*, and *P. aeruginosa*) were evaluated by assessing extracellular enzyme production using standard microbiological methods [20]. The virulence factors investigated included hemolysin, lecithinase, lipase, caseinase, gelatinase, amylase, DNase, and esculinase.

Hemolysin production was assessed on 5% sheep blood agar following incubation at 37°C for 24 h, with clear zones surrounding colonies indicating β-haemolysis. Lecithinase activity was determined on egg yolk agar, where opalescent halos surrounding colonies were considered positive. Lipase production was evaluated on Rhodamine agar after incubation at 37°C for 1–5 days. Caseinase activity was assessed on skim milk agar by observing zones of casein hydrolysis. Gelatinase production was determined using nutrient gelatin medium, with liquefaction after refrigeration indicating a positive reaction. Amylase activity was evaluated on starch agar following iodine staining, while DNase activity was determined using DNase agar based on DNA hydrolysis around bacterial growth. Esculinase activity was assessed on bile esculin agar, where blackening of more than half of the medium was considered positive.

Biofilm formation assay

Biofilm-forming ability of the MDR isolates (*A. baumannii, E. coli, K. pneumoniae, P. aeruginosa*, and MRSA) was evaluated using the crystal violet microtiter plate assay with minor modifications of the method described by Awuor *et al.* [20]. Overnight cultures were adjusted to a 0.5 McFarland standard (approximately 1×10^8 CFU/mL) in tryptic soy broth (TSB). Aliquots (20 µL) of each bacterial suspension were inoculated into wells containing 180 µL TSB in sterile 96-well microplates and incubated at 37°C for 24, 48, and 72 h. Each isolate was tested in triplicate in three independent experiments.

Following incubation, planktonic cells were removed by washing the wells three times with phosphate-buffered saline (PBS). Adherent biofilms were fixed with cold methanol, stained with 0.1% crystal violet for 15 min, washed thoroughly, and air-dried. Biofilm biomass was quantified by measuring absorbance at 590 nm using a Tecan Safire microplate reader.

Biofilm inhibition was calculated using the following equation:

$$\text{Biofilm inhibition (\%)} = \left[1 - \frac{OD_{\text{test}} - OD_{\text{blank}}}{OD_{\text{untreated}} - OD_{\text{blank}}}\right] \times 100$$

DNA extraction and quality assessment

Genomic DNA was extracted from purified MDR isolates using the boiling lysis method with minor modifications [28]. Briefly, several colonies from overnight cultures grown on Mueller–Hinton agar were suspended in 100 µL of PCR-grade water, heated at 95°C for 10 min, cooled on ice for 30 min, and centrifuged at 15,000 rpm for 3 min. The resulting supernatant containing genomic DNA was transferred to sterile microcentrifuge tubes and stored at − 20°C until molecular analysis.

DNA concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Scientific, USA). DNA samples with A260/A280 ratios between 1.8 and 2.0 were considered suitable for PCR amplification [21].

PCR detection of antimicrobial resistance genes

PCR analysis was performed only on the nineteen MDR isolates. Phenotypically confirmed MRSA isolates were screened for the **mecA** gene. Gram-negative MDR isolates exhibiting resistance to third-generation cephalosporins and/or phenotypic ESBL production were screened for the **blaTEM**, **blaSHV**, **blaCTX-M-1**, and **blaKPC** genes.

PCR amplification was performed in a final reaction volume of 25 µL containing 12.5 µL Hot Start Taq 2× Master Mix (New England Biolabs), 1.0 µL each of forward and reverse primers (10 µM), 5.0 µL DNA template, and 5.5 µL nuclease-free water.

Amplification was carried out using a CLASSIC K960 thermal cycler under the following conditions: initial denaturation at 95°C for 1 min; 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for **mecA**, **blaTEM**, **blaSHV**, and **blaKPC** or 62°C for **blaCTX-M-1** for 1 min, and extension at 72°C for 1 min; followed by a final extension at 72°C for 5 min.

PCR products were separated by electrophoresis on a 1.5% agarose gel stained with an appropriate nucleic acid stain and visualized under UV illumination. Expected amplicon sizes were determined by comparison with a 100-bp DNA ladder. Primer sequences and expected amplicon sizes are presented in Table 1.

Table 1: Primer sequences and PCR conditions are presented in this study

Genes	Oligonucleotide sequences (5' to 3')	Size (Bp)	Corresponding organism.	PCR conditions			References
				Melting	Annealing	Extension	
***bla*$_{SHV}$**	GGTTATGCGTTATATTCGCC TTAGCGTTGCCAGTGCTC	867	*E. coli* *K. pneumoniae*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[22]
***bla*$_{OXA}$**	ACACAATACATATCAACTTCGC AGTGTGTTTAGAATGGTGATC	885	*A. baumannii-*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[23]
***bla*$_{VIM}$**	CTTCATTCACGCACTATTAC TAACTTGACCGACAGAGG	827	*P.aeruginosa*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[24]
blaCTX-M-1	ATTCGTATGCTGGATCTCGCCACC CATGACCCAGTTCGCCATATCCTG	396	*P.aeruginosa*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[25]
***bla*$_{VEB}$**	CGACTTCCATTTCCCGATGC GGACTCTGCAACAAATACGC	1014	*P.aeruginosa*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[26]
***bla*$_{SPM-1}$**	GTACAAGGGATTCGGCATCG TGGCCTGTTCCCATGTGAG	569	*P.aeruginosa*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[27]
***bla*$_{KPC}$**	TCTGGACCGCTGGGAGCTGG TGCCCGTTGACGCCCAATCC		*Enterococci spp.* *E. coli* *K. pneumoniae*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[28]
Mex-A	CTCGACCCGATCTACGTC GTCTTCACCTCGACACCC	503	*P.aeruginosa*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[22]
Mex-B	TGTCGAAGTTTTTCATTGAG AAGGTCAC GGTGATGGT	280	*P.aeruginosa*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[22]
Mex-R	GAACTACCCCGTGAATC CACTGGTCGAGGAGATGC	411	*P.aeruginosa*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[22]
OprM	GATCCCCGACTACCAGCGCCCCG ATGCGGTACTGCGCCCGGAAGGC	247	*Enterococci spp.* *E. coli* *K. pneumoniae*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[22]
OprD	ATCTACCGCACAAACGATGAG GCCGAAGCCGATATAATCAAACG	156	*P.aeruginosa*	95 ^{0}C, 1 min	58 ^{0}C, 1 min	72 ^{0}C, 1 min	[22]
mecA	TCCAGATTACAACTTCACCAGG CCACTTCATATCTTGTAACG	162	*S. aureus*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[28]
vanA	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	1030	*E. coli*	95 ^{0}C, 1 min	58 ^{0}C, 1 min	72 ^{0}C, 1 min	[29]
vanB	GTGACAAACCGGAGGCGAGGA CGCCATCCTCCTGCAAAAAA	433	*Enterococci spp.*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[23]
vanC	ATCCAAGCTATTGACCCGCT TGTGGCAGGATCGTTTTCAT	402	*Enterococci spp.*	95 ^{0}C, 1 min	54 ^{0}C, 1 min	72 ^{0}C, 1 min	[30]

Gel electrophoresis

PCR amplicons were separated by electrophoresis on a 1.5% (w/v) agarose gel prepared in 1× Tris–borate–EDTA (TBE) buffer and stained with SafeView Classic™ DNA stain (Cat. No. G108) as previously described [31]. Prior to loading, each PCR product was mixed with 6× DNA loading dye (Thermo Scientific, Cat. No. R0611). Approximately 20 µL of each PCR product, together with positive and negative controls, was loaded onto the gel alongside a 100-bp DNA ladder (New England Biolabs, Cat. No. N3231L).

Electrophoresis was performed in 1× TBE running buffer at 200 V and 80 mA for 60 min. The amplified DNA fragments were visualized under ultraviolet illumination using a GeneFlash gel documentation system, and images were captured for analysis. Amplicon sizes were determined by comparison with the 100-bp DNA ladder.

Ethics approval and consent to participate

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Ethical approval was obtained from the Institutional Research Ethics Committee (IREC) of Jaramogi Oginga Odinga Teaching and Referral Hospital (Ref. No. ISERC/JOOTRH/096/2024). Additional approvals were obtained from the National Commission for Science, Technology and Innovation (Ref. No. NACOSTI/P/25/16381) and the Kisumu County Ministries of Health and Education (Ref. No. GN 133 VOL. XIX/168).

Written informed consent was obtained from all participants before enrolment. Participant confidentiality and privacy were maintained throughout the study by assigning unique study identification numbers and excluding all personal identifiers from laboratory records, databases, and publications.

Data Management and Analysis All laboratory experiments were performed in triplicate and independently to ensure the reproducibility and reliability of the results. Laboratory data were initially recorded in standardized laboratory notebooks and subsequently entered Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) for data cleaning, validation, and organization. The cleaned dataset was exported to GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) for statistical analysis and graphical presentation.

Quantitative data are presented as the mean ± standard error of the mean (SEM) from three independent experiments. Statistical differences among groups were evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test, with the untreated control group serving as the reference for all pairwise comparisons. A two-tailed P value < 0.05 was considered statistically significant.

Results

Among the nine multidrug-resistant (MDR) Gram-positive isolates, 5 (55.6%) produced at least one of the virulence factors evaluated, including hemolysin, lecithinase, lipase, and amylase. The remaining 4 (44.4%) isolates did not produce any of the tested virulence factors under the experimental conditions. The distribution of virulence factor production among the Gram-positive isolates is presented in Table 2.

Table 2: Virulence factors production among the MDR Gram-positive pathogens isolated from DFU patients.

Isolate Numbers	Virulence Factors							
Gram- Positive Isolates	***Hemolysin***	***Lecithinase***	***Lipase***	***Caseinase***	***Gelatine***	***Amylase***	***DNase***	***Esculinase***
• *LSCH/KC/34*	+	+	+	-	-	+	-	-
• *KCRH/KC/132*	-	-	-	-	-	-	-	-
• *JOOTRH/KC/103*	-	-	-	-	-	-	-	-
• *JOOTRH/KC/96*	+	+	+	-	-	+	-	-
• *JOOTRH/KC/113*	+	+	+	-	-	+	-	-
• *JOOTRH/KC/59*	-	-	-	-	-	-	-	-
• *KCRH/KC/103*	+	+	+	-	-	+	-	-
• *JOOTRH/KC/49*	+	+	+	-	-	+	-	-
• *LSCH/KC/46*	-	-	-	-	-	-	-	-

Keys: [+], Positive and **[-],** Negative, *Staphylococcus aureus* isolates (LCSH/KC/34, KCRH/KC/132, JOOTRH/KC/103, JOOTRH/KC/96, JOOTRH/KC/49, JOOTRH/KC/113, JOOTR/KC/59, LCSH/KC/46, and KCRH/KC/103)

Among the ten multidrug-resistant (MDR) Gram-negative isolates, comprising *Escherichia coli* (n = 2), *Acinetobacter baumannii* (n = 5), *Klebsiella pneumoniae* (n = 2), and *Pseudomonas aeruginosa* (n = 1), **five isolates (50.0%)** produced all the virulence factors evaluated, while the remaining **five isolates (50.0%)** produced only a subset of the tested virulence factors (Table 3).

Table 3: Virulence factors production among the MDR Gram-negative pathogens isolated from DFU patients.

Isolate Numbers	Virulence Factors							
Gram-negative Isolates	**Hemolysin**	**Lecithinase**	**Lipase**	**Caseinase**	**Gelatine**	**Amylase**	**DNase**	**Esculinase**
• JOOTRH/KC/49i	+	+	+	+	+	+	+	+
• KCRH/KC/29i	+	+	+	+	+	+	+	+
• JOOTRH/KC/45	-	-	-	-	-	-	-	-
• JOOTRH/KC/35	+	+	+	-	-	+	-	-
• KCRH/KC/43	+	+	+	+	+	+	+	+
• LSCH/KC/41	-	-	-	-	-	-	-	-
• LSCH/KC/23	+	+	+	-	-	+	-	-
• JOOTRH/KC/44	+	+	+	+	+	+	+	+
• KCRH/KC/50	-	-	-	-	-	-	-	-
• JOOTRH/KC/94	+	+	+	+	+	+	+	+

Keys: [+], Positive and **[-],** Negative, *Escherichia coli* (JOOTRH/KC/49[i], KCRH/KC/29[i]), *Acinetobacter baumannii* (JOOTRH/KC/45, JOOTRH/KC/35, KCRH/KC/43, LSCH/KC/41, and LSCH/KC/23), *Klebsiella pneumoniae* (JOOTRH/KC/44 and KCRH/KC/50), and *Pseudomonas aeruginosa* (JOOTRH/KC/94)

Antibiofilm activity of selected antibiotics

The antibiotics selected for antibiofilm evaluation were those that exhibited the highest resistance rates among the multidrug-resistant (MDR) isolates during antimicrobial susceptibility testing. Only MDR isolates that demonstrated production of one or more virulence factors were included in the antibiofilm assay. Seven MDR isolates were therefore evaluated, comprising *Escherichia coli* (JOOTRH-KC-49[i]), *Klebsiella pneumoniae* (JOOTRH-KC-44), *Pseudomonas aeruginosa* (JOOTRH-KC-94), *Acinetobacter baumannii* (KCRH-KC-43), and three *Staphylococcus aureus* isolates (KCRH-KC-103, LSCH-KC-34, and JOOTRH-KC-113).

Two-fold serial dilutions of each antibiotic were prepared to obtain final concentrations ranging from 1.0 to 0.0625 mg/mL. Biofilm inhibition was assessed using the crystal violet microtiter plate assay. *P. aeruginosa* ATCC 10145 served as the positive control, untreated bacterial cultures served as negative controls, and uninoculated wells containing culture medium alone were included as blanks for background correction.

Overall, all antibiotics tested reduced biofilm formation in a concentration-dependent manner. However, the magnitude of inhibition varied among bacterial species and antibiotics. One-way ANOVA followed by Dunnett's multiple comparisons test demonstrated significant reductions in biofilm biomass for selected antibiotic concentrations compared with the untreated controls (Figures 1 A-G). Despite these reductions, all seven MDR isolates retained the ability to form biofilms, indicating persistence of the biofilm-forming phenotype.

For *E. coli* (JOOTRH-KC-49[i]), imipenem, levofloxacin, and ciprofloxacin exhibited the greatest antibiofilm activity, whereas ofloxacin and amoxicillin-clavulanate showed comparatively weaker effects (Figure 1A). Significant inhibition of biofilm formation was observed at selected concentrations, with ciprofloxacin demonstrating relatively limited antibiofilm activity despite its widespread clinical use.

Klebsiella pneumoniae (JOOTRH-KC-44) exhibited limited susceptibility to the antibiofilm effects of most antibiotics. Nevertheless, ciprofloxacin significantly reduced biofilm formation at 0.25 mg/mL ($P = 0.001$) and 0.50 mg/mL ($P < 0.05$) (Figure 1B).

For *P. aeruginosa* (JOOTRH-KC-94), antibiofilm activity was generally modest across all antibiotics tested. Greater inhibition was observed at higher antibiotic concentrations (1.0, 0.50, and 0.25 mg/mL), while lower concentrations produced minimal effects (Figure 1C).

Among *A. baumannii* (KCRH-KC-43), antibiofilm activity varied depending on the antibiotic tested. Amoxicillin-clavulanate showed no statistically significant reduction in biofilm formation at any of the concentrations evaluated compared with the untreated control (Figure 1D).

For *S. aureus* isolate KCRH-KC-103, levofloxacin, azithromycin, cefoxitin, and chloramphenicol significantly inhibited biofilm formation at 0.125 mg/mL ($P < 0.01$) (Figure 1E). Similarly, *S. aureus* isolate LSCH-KC-34 demonstrated significant biofilm inhibition following treatment with levofloxacin at 0.125 mg/mL and 0.0625 mg/mL ($P = 0.001$) (Figure 1F). For isolate JOOTRH-KC-113, tetracycline produced significant antibiofilm activity at 0.125 mg/mL and 0.0625 mg/mL ($P = 0.001$), whereas the remaining antibiotics produced limited or no significant inhibition (Figure 1G).

Characterization of the *mecA* Gene in *Staphylococcus aureus*

Nine multidrug-resistant (MDR) *Staphylococcus aureus* isolates (LSCH/KC/34, KCRH/KC/132, JOOTRH/KC/103, JOOTRH/KC/96, JOOTRH/KC/49, JOOTRH/KC/113, JOOTRH/KC/59, LSCH/KC/46, and KCRH/KC/103) exhibiting phenotypic resistance to multiple antimicrobial classes, including quinolones, sulfonamides, oxazolidinones, and macrolides, were screened for the *mecA* gene by uniplex PCR. Amplification of the target gene produced the expected amplicon size of 147 bp.

The *mecA* gene was detected in five (55.6%) of the nine MDR *S. aureus* isolates, specifically JOOTRH/KC/96, JOOTRH/KC/113, JOOTRH/KC/49, KCRH/KC/103, and LSCH/KC/34 (Figure 2, Supplementary 1). The remaining four isolates (44.4%) were negative for *mecA*, suggesting that methicillin resistance in these isolates may be mediated by alternative genetic or regulatory mechanisms.

Characterization of β-Lactamase Genes in Selected MDR Gram-Negative Bacteria

Ten multidrug-resistant (MDR) Gram-negative isolates comprising *Escherichia coli* (JOOTRH/KC/49[i] and KCRH/KC/29[i]), *Acinetobacter baumannii* (JOOTRH/KC/45, JOOTRH/KC/35, KCRH/KC/43, LSCH/KC/41, and LSCH/KC/23), *Klebsiella pneumoniae* (JOOTRH/KC/44 and KCRH/KC/50), and *Pseudomonas aeruginosa* (JOOTRH/KC/94) were screened for the β-lactamase genes blaTEM, blaKPC, blaCTX-M-1, and blaSHV by uniplex PCR. All isolates exhibited phenotypic resistance to cefixime, a third-generation cephalosporin, in addition to resistance to multiple antimicrobial classes.

No amplification was detected for blaTEM, blaKPC, or blaSHV in any of the isolates tested. In contrast, amplification of blaCTX-M-1 yielded the expected 499-bp PCR product. Overall, five (50.0%) of the ten MDR Gram-negative isolates were positive for blaCTX-M-1, including JOOTRH/KC/49(i), KCRH/KC/29(i), KCRH/KC/43, JOOTRH/KC/94, and KCRH/KC/50(Figure 3, Supplementary 2).

Discussion

In this study, nineteen multidrug-resistant (MDR) bacterial isolates recovered from infected diabetic foot ulcers (DFUs) were further characterized to determine their virulence profiles, antibiofilm activity, and molecular resistance determinants. The findings demonstrate that MDR pathogens circulating in Kisumu County possess clinically important virulence traits and resistance genes that may contribute to persistent infections, delayed wound healing, and poor therapeutic outcomes in patients with DFUs. The coexistence of virulence factors, biofilm-forming capacity, and antimicrobial resistance underscores the complexity of managing diabetic foot infections and highlights the need for continuous microbiological and molecular surveillance.

Virulence factors play a central role in bacterial colonization, tissue invasion, immune evasion, and persistence within chronic wounds. In the present study, 5/9 (55.6%) Gram-positive MDR isolates produced at least one virulence factor, including hemolysin, lecithinase, lipase, and amylase, while 5/10 (50.0%) Gram-negative isolates expressed all the virulence factors evaluated. These findings are comparable to those reported previously in Kisumu County, where 62% of MDR wound isolates produced virulence factors [15], and to reports describing a 57% prevalence among *Acinetobacter baumannii* isolates recovered from hospital environments [33]. Similar observations have also been made in Uganda, where expression of multiple virulence determinants among MDR isolates was associated with increased pathogenic potential and persistence in healthcare settings [34]. Collectively, these findings suggest that extracellular enzymes may facilitate tissue destruction, nutrient acquisition, and immune evasion, thereby contributing to chronic infection and delayed healing of diabetic foot ulcers.

Biofilm formation is an important mechanism by which bacteria tolerate antimicrobial therapy and evade host immune responses, contributing to the persistence of chronic diabetic foot infections. In the present study, all seven-virulence factor-producing MDR isolates retained the ability to form biofilms despite exposure to multiple antibiotics. Interestingly, several antibiotics, including levofloxacin, ciprofloxacin, tetracycline, cefoxitin, imipenem, azithromycin, and amoxicillin-clavulanate, demonstrated greater inhibition of biofilm formation at lower concentrations than at higher concentrations. Similar non-linear concentration-dependent responses have been reported previously and described as the "Goldilocks effect," whereby intermediate or lower antimicrobial concentrations appear to produce greater antibiofilm activity than higher concentrations [35,36]. However, the underlying mechanisms responsible for this phenomenon remain poorly understood and were not investigated in the present study. Therefore, the observed concentration-dependent differences should be interpreted cautiously and regarded as preliminary observations that require confirmation through mechanistic studies.

Our findings differ from those of previous studies reporting increased antibiofilm activity of azithromycin and fluoroquinolones with increasing antibiotic concentrations [37], suggesting that antibiofilm responses may vary according to bacterial species, strain characteristics, antibiotic class, and experimental conditions. Despite measurable reductions in biofilm formation, none of the antibiotics completely inhibited biofilm development, emphasizing the persistence of biofilm-associated bacterial communities and highlighting the need for further research into combination therapies, novel antibiofilm agents, and optimized treatment strategies for chronic diabetic foot infections.

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains one of the most clinically significant pathogens associated with diabetic foot infections because of its resistance to nearly all β-lactam antibiotics. In the present study, the mecA gene was detected in 5/9 (55.6%) phenotypically resistant *S. aureus* isolates. This prevalence is comparable to that reported in Saudi Arabia

(53.8%) [12] but lower than those reported in Mexico (96.8%), Sudan (90.2%), and Indonesia (100%) [38,39]. Conversely, it is higher than the prevalence reported in Uganda (46%) and that observed in a recent global meta-analysis (21.7%) [19]. Previous studies conducted in Kisumu County have similarly documented the circulation of mecA-positive *S. aureus* isolates [9,20], supporting the continued presence of MRSA in the region.

Detection of the mecA gene provides molecular confirmation of methicillin resistance through the production of the altered penicillin-binding protein PBP2a. However, four phenotypically resistant isolates lacked detectable mecA, indicating that methicillin resistance may also result from alternative mechanisms, including hyperproduction of β-lactamase, alterations in native penicillin-binding proteins, or the presence of other mec homologues such as mecC, which were not investigated in the present study. This discrepancy highlights the limitations of relying solely on phenotypic susceptibility testing and reinforces the value of incorporating molecular diagnostics into routine clinical microbiology to improve diagnostic accuracy and support antimicrobial stewardship programmes [40].

Among the MDR Gram-negative isolates, phenotypic evidence of extended-spectrum β-lactamase (ESBL) production was observed in 6/10 (60.0%) isolates based on cefixime resistance and confirmation using the cefixime-clavulanate combined disk test. Molecular characterization revealed that six isolates carried the blaCTX-M-1 gene, whereas only one isolate harboured blaSHV. No isolates carried blaTEM or blaKPC. CTX-M enzymes are currently the predominant ESBLs reported worldwide and are strongly associated with resistance to third-generation cephalosporins, making them important drivers of multidrug resistance among Gram-negative pathogens.

The prevalence of blaCTX-M-1 observed in this study is similar to that reported in the Gaza Strip (60%) [41] but higher than reports from China (34.7%), Nepal (49.3%), and Bangladesh (20.7%) [42–44]. However, it remains lower than the 100% prevalence reported among clinical isolates in Jos, Nigeria [45]. These geographical differences are likely influenced by variations in antimicrobial prescribing practices, infection prevention measures, healthcare infrastructure, and local epidemiological patterns. The predominance of blaCTX-M-1 among MDR isolates in this study supports previous evidence that CTX-M enzymes have largely replaced earlier TEM-derived ESBLs as the dominant mechanism of cephalosporin resistance worldwide.

Only one *Acinetobacter baumannii* isolate (KCRH/KC/43) carried the bla_{SHV} gene, and this isolate simultaneously harboured blaCTX-M-1, demonstrating co-occurrence of multiple β-lactamase genes within a single MDR isolate. Co-carriage of multiple ESBL genes may broaden the spectrum of β-lactam resistance and facilitate horizontal dissemination of resistance determinants, thereby increasing therapeutic challenges. The relatively low prevalence of blaSHV observed in this study suggests that SHV-type β-lactamases currently play a less prominent role than CTX-M enzymes in mediating β-lactam resistance among DFU-associated pathogens in Kisumu County.

The absence of blaTEM and blaKPC contrasts with previous reports from Kenya, where these genes have been detected, albeit at variable frequencies [9,18]. Their absence in the present study may indicate that resistance among MDR Gram-negative isolates in Kisumu County is currently driven predominantly by CTX-M-type ESBLs rather than TEM-type ESBLs or KPC carbapenemases. Alternatively, these findings may reflect differences in patient populations, antimicrobial selective pressure, healthcare settings, or geographical variation in circulating resistance determinants. Nevertheless, the observed heterogeneity reinforces the importance of continued molecular surveillance to monitor evolving resistance mechanisms and guide empirical antimicrobial therapy.

This study has several limitations. First, molecular characterization was restricted to nineteen MDR isolates because of resource constraints, which may have underestimated the diversity of resistance genes circulating among DFU pathogens. Second, only selected resistance determinants (mecA, blaCTX-M-1, blaSHV, blaTEM, and blaKPC) were investigated, while other clinically relevant genes such as blaNDM, blaOXA-48-like, blaVIM, blaIMP, and mecC were not evaluated. Third, whole-genome sequencing was not performed, limiting comprehensive characterization of resistance and virulence determinants. Despite these limitations, this study provides important baseline molecular epidemiological data on MDR pathogens associated with diabetic foot infections in western Kenya.

Overall, the findings demonstrate that MDR bacterial pathogens associated with diabetic foot ulcers in Kisumu County possess important virulence factors, maintain substantial biofilm-forming capacity despite antimicrobial exposure, and predominantly harbour CTX-M-mediated β-lactam resistance. These results highlight the need for routine molecular surveillance, strengthened

antimicrobial stewardship, and continued investigation of alternative therapeutic strategies targeting both antimicrobial resistance and biofilm-associated persistence to improve the clinical management of diabetic foot infections.

Conclusion

This study demonstrates that multidrug-resistant bacterial pathogens associated with diabetic foot ulcers in Kisumu County possess important virulence characteristics, biofilm-forming ability, and clinically relevant antimicrobial resistance genes, highlighting the complexity of managing these infections. The findings emphasize the value of integrating molecular diagnostics with conventional microbiological methods to improve the detection of antimicrobial resistance and support evidence-based treatment decisions. Strengthening antimicrobial stewardship, routine molecular surveillance, and infection prevention and control measures will be essential to limit the emergence and spread of multidrug-resistant pathogens. Further studies incorporating larger sample sizes and comprehensive genomic analyses are warranted to better understand the epidemiology and transmission of resistance determinants in diabetic foot infections.

Study Limitations

This study has several limitations. First, molecular characterization was limited to a relatively small number of multidrug-resistant (MDR) isolates, which may limit the generalizability of the findings. Second, only selected antimicrobial resistance genes (mecA, blaCTX-M-1, blaSHV, blaTEM, and blaKPC) were investigated, and other clinically relevant resistance determinants were not assessed. Third, the antibiofilm assays were performed under in vitro conditions, which may not fully reflect the complex host environment encountered during diabetic foot infections. Finally, whole-genome sequencing and molecular typing were not performed, limiting comprehensive characterization of resistance mechanisms, virulence determinants, and bacterial relatedness.

Future Perspectives

Future studies should include larger multicentre cohorts to better define the epidemiology of multidrug-resistant pathogens associated with diabetic foot infections. The application of whole-genome sequencing and other high-resolution molecular approaches would enable comprehensive characterization of resistance genes, virulence factors, mobile genetic elements, and transmission dynamics. In addition, in vivo studies are needed to validate the antibiofilm activity observed in vitro and to evaluate novel therapeutic strategies, including combination therapies and anti-biofilm agents. Continued molecular surveillance, together with strengthened antimicrobial stewardship and infection prevention programmes, will be essential for improving the management and control of multidrug-resistant diabetic foot infections.

Declarations

Competing interests

The authors declare no competing interests.

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Author Contribution

Conceptualization: SAO, EOO. Methodology: SAO, EOO, EW, IID. Resources: SAO. Writing—original draft: SAO, RMM. Writing—review and editing: SAO, EOO, EW, IID, RMM. Visualization: SAO, EOO. Supervision: EOO, EW, IID. Project administration: SAO. Final editing: all authors. All authors have read and approved the final version of the manuscript.

Data availability statement

All the data has been shared in the manuscript

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Figures

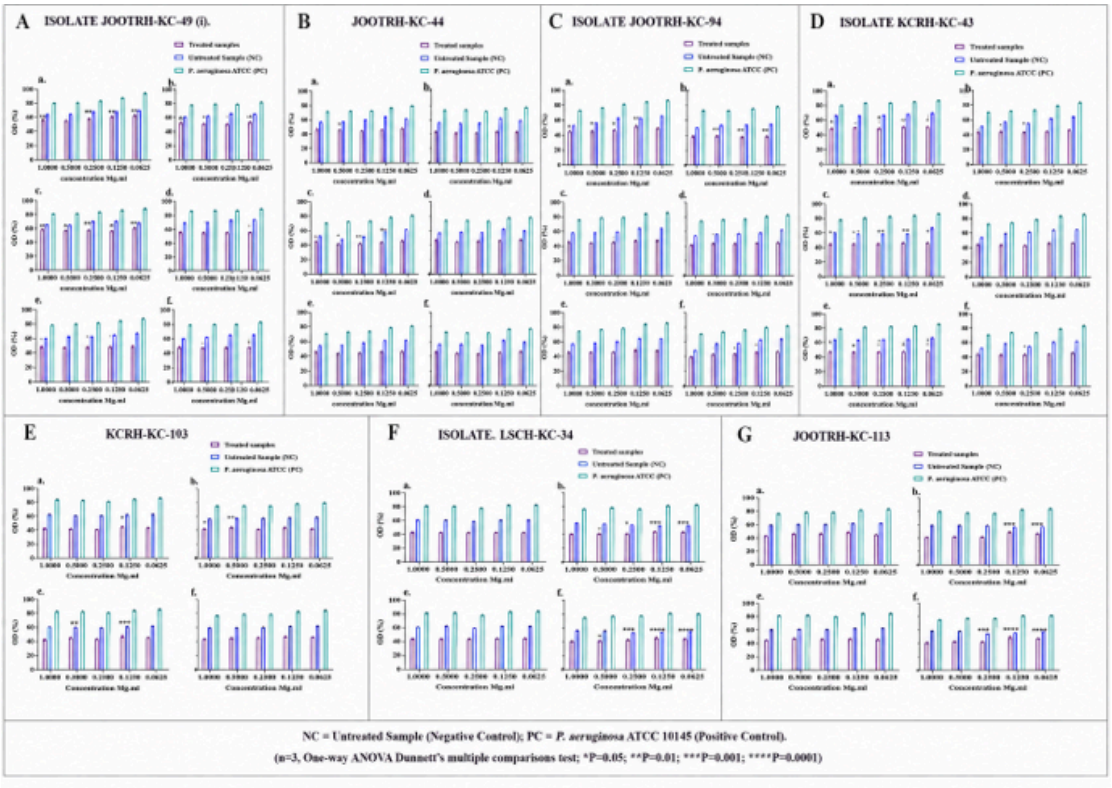


Figure 1

Antibiofilm activity of selected antibiotics against multidrug-resistant (MDR) bacterial isolates recovered from diabetic foot ulcers. Biofilm inhibition was evaluated using the crystal violet microtiter plate assay following exposure to two-fold serial dilutions of selected antibiotics. Panels represent the antibiofilm activity against (A) Escherichia coli isolate JOOTRH-KC-49(i), (B) Klebsiella pneumoniae isolate JOOTRH-KC-44, (C) Pseudomonas aeruginosa isolate JOOTRH-KC-94, (D) Acinetobacter baumannii isolate KCRH-KC-43, (E) Staphylococcus aureus isolate KCRH-KC-103, (F) Staphylococcus aureus isolate LSCH-KC-34, and (G) Staphylococcus aureus isolate JOOTRH-KC-113. Panels A–D show the effects of imipenem, levofloxacin, ciprofloxacin, ofloxacin, cefoxitin, and amoxicillin–clavulanate, whereas panels E–G show the effects of tetracycline, levofloxacin, ciprofloxacin, azithromycin, cefoxitin, and chloramphenicol. Untreated bacterial cultures served as the negative control (NC), while

Pseudomonas aeruginosa ATCC 10145 served as the positive control (PC). Data are presented as the mean ± standard error of the mean (SEM) from three independent experiments (n = 3). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test, with comparisons made against untreated control (*P < 0.05, **P < 0.01, ***P < 0.001, ***P < 0.0001).

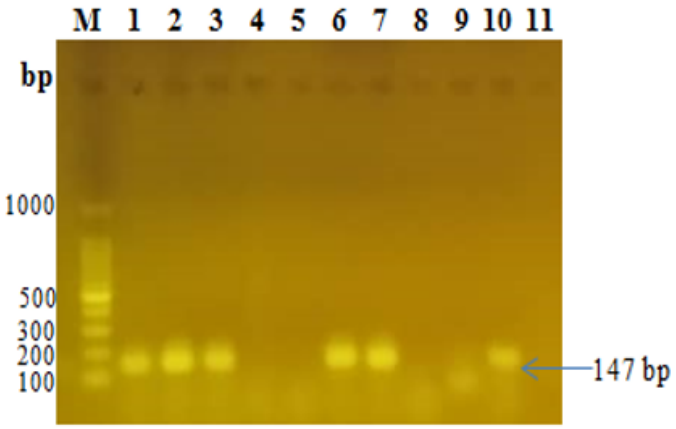


Figure 2

Agarose gel electrophoresis of PCR amplicons showing detection of the mecA gene (147 bp) in selected multidrug-resistant (MDR) Staphylococcus aureus isolates recovered from diabetic foot infection (DFI) wounds. Lane M: 100-bp DNA ladder; Lane 1: JOOTRH/KC/96; Lane 2: JOOTRH/KC/113; Lane 3: JOOTRH/KC/49; Lane 4: KCRH/KC/132; Lane 5: JOOTRH/KC/103; Lane 6: KCRH/KC/103; Lane 7: LSCH/KC/34; Lane 8: JOOTRH/KC/59; Lane 9: LSCH/KC/46; Lane 10: positive control (S. aureus ATCC 25923); Lane 11: negative control.

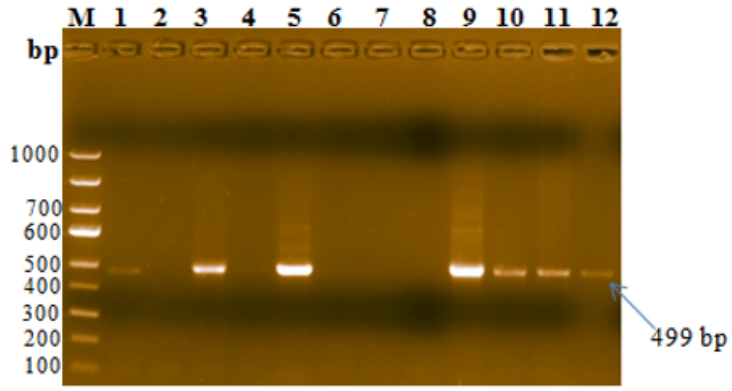


Figure 3

Agarose gel electrophoresis of PCR amplicons showing detection of the blaCTX-M-1 gene (499 bp) in selected multidrug-resistant (MDR) Gram-negative bacterial isolates recovered from diabetic foot infection (DFI) wounds. Lane M: 100-bp DNA ladder; Lane 1: KCRH/KC/50 (Klebsiella pneumoniae); Lane 2: JOOTRH/KC/35 (Acinetobacter baumannii); Lane 3: positive control (Escherichia coli ATCC 25922); Lane 4: negative control; Lane 5: JOOTRH/KC/44 (Klebsiella pneumoniae); Lane 6: JOOTRH/KC/45 (Acinetobacter baumannii); Lane 7: LSCH/KC/23 (Acinetobacter baumannii); Lane 8: LSCH/KC/41 (Acinetobacter baumannii); Lane 9: JOOTRH/KC/94 (Pseudomonas aeruginosa); Lane 10: JOOTRH/KC/49(i) (Escherichia coli); Lane 11: KCRH/KC/43 (Acinetobacter baumannii); and Lane 12: KCRH/KC/29(i) (Escherichia coli).PCR amplification of the blaSHV gene yielded the expected amplicon size of 201 bp. Among the ten MDR Gram-negative isolates screened, one isolate (10.0%), KCRH/KC/43 (Acinetobacter baumannii), was positive for blaSHV(Figure 10). This isolate also carried the blaCTX-M-1 gene, indicating the co-occurrence of multiple β-lactamase genes (Figure 4, Supplementary 3)

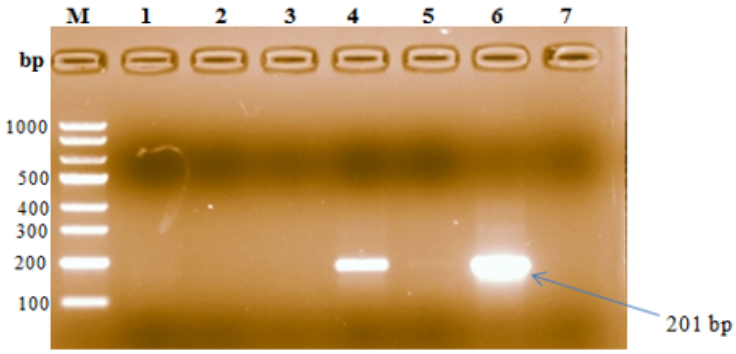


Figure 4

Agarose gel electrophoresis of PCR amplicons showing detection of the blaSHV gene (201 bp) in selected multidrug-resistant (MDR) Gram-negative bacterial isolates recovered from diabetic foot infection (DFI) wounds. Lane M: 100-bp DNA ladder; Lane 1: LSCH/KC/41 (*Acinetobacter baumannii*); Lane 2: LSCH/KC/23 (Acinetobacter baumannii); Lane 3: JOOTRH/KC/45 (*Acinetobacter baumannii*); Lane 4: positive control (*Acinetobacter baumannii* ATCC 19606); Lane 5: non-template control (NTC); Lane 6: KCRH/KC/43 (*Acinetobacter baumannii*); and Lane 7: JOOTRH/KC/35 (*Acinetobacter baumannii*). The expected amplicon size for the blaSHV</sub> gene was 201 bp.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SuplementaryData1.xlsx
- Supplimentary1forfig.8.png
- Suplementary2.forFig.9.png
- Supplimentary3Fig.10.png