



Bacterial Profile and Multiplex PCR Detection of SHV, CTX-M, and mecA Genes in Diabetic Ulcer Infections

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Abstract: Diabetes mellitus is a chronic disease with complications such as diabetic ulcers, which can lead to amputation. High blood glucose facilitates a mix of aerobic and anaerobic bacterial growth in ulcers. Prolonged antibiotic use risks resistance. This study aims to identify bacterial species and the presence of resistance genes (SHV, CTX-M, and mecA) in isolates from diabetic ulcer patients at Wahidin Hospital, Mojokerto, using multiplex PCR. The study design was a descriptive, observational study to identify bacterial patterns and detect the presence of SHV, CTX-M, and mecA genes in bacterial samples from ulcers of diabetic patients using a multiplex molecular genetic method. Bacterial identification of 32 ulcer swab samples from DM patients revealed *Staphylococcus aureus* (63%), *Escherichia coli* (28%), and *Klebsiella pneumoniae* (9%). The results of the detection of multiplexed genes encoding resistance in the identified bacteria were SHV 3%, CTX-M 19%, mecA 22%, SHV & mecA 3%, and CTX-M & mecA 3%. The multiplex PCR method was successfully used to detect genes encoding antibiotic resistance in bacteria infecting ulcers of patients with DM.

Keywords: CTX-M; diabetic ulcers; mecA; Multiplex Polymerase Chain Reaction; SHV.

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease characterized by elevated glucose levels due to reduced insulin secretion by the pancreas and is a serious health problem with the risk of causing several other disease complications (Fau et al., 2021). Complications that occur in people with diabetes include damage to the kidneys, brain, heart, eyes, and feet, known as diabetic ulcers (Anggraini et al., 2020). According to the International Diabetes Federation (IDF) Atlas 10th Edition (2021) and updated projections for 2023-2024, Indonesia has risen to the 5th highest position globally, with approximately 19.5 to 20.4 million people living with diabetes. This number is projected to reach 28.6 million by 2045 (IDF, 2024). Elevated blood glucose levels can lead to a 40% risk of non-healing foot ulcers, characterized by the formation of lesions that can lead to amputation (Matheson et al., 2021). Hyperglycemia is a fertile environment for the growth of aerobic and anaerobic bacteria (Matheson et al., 2021).

Globally, bacterial patterns in DFUs have shifted toward a dominance of Gram-negative pathogens in Asian regions, contrasting with the Gram-positive prevalence often seen in Western countries (Selano et al., 2021). In Indonesia, while studies in major metropolitan areas have identified various multidrug-resistant (MDR) strains, specific molecular data and bacterial mapping in smaller regions like Mojokerto remain significantly underreported.

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Bacteria found in diabetic wounds are a combination of aerobic and anaerobic bacteria (Sartika et al., 2020). Based on previous research, the most common bacteria found in diabetic ulcers were 79.6% gram-negative bacteria and 20.4% gram-positive bacteria (Jain & Barman, 2017). In Indonesia, research on bacterial patterns and resistance among diabetic ulcer patients in Surabaya and Padang shows that *Escherichia coli* (33.3%) and *Klebsiella pneumoniae* (27%–40%) are the most dominant pathogens. Other identified bacteria include *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Proteus* species. The data indicate a decline in antibiotic effectiveness for these patients, with the highest resistance rates (40%) observed against ciprofloxacin and cefotaxime, followed by meropenem, gentamicin, and vancomycin (30%) (Donastin & Aisyah, 2019; Novelni, 2019).

β -lactam antibiotics play a crucial role in modern medical practice, accounting for approximately two-thirds of all hospital prescriptions. However, the efficacy of this antibiotic class is severely compromised by bacterial resistance, primarily driven by the production of β -lactamase enzymes that deactivate the antibiotic molecules. Consequently, the World Health Organization (WHO) has classified β -lactamase-producing Gram-negative bacteria as one of the most critical global health threats (Bush K, Bradford PA. 2020). The colonization of these pathogens is no longer confined to clinical settings but has extended to healthcare centers and the general population. At the molecular level, drug resistance is mediated by specific genetic determinants, such as the SHV, CTX-M, and *mecA* genes, which have been identified in bacteria infecting diabetic ulcers (Ghenea et al., 2022). Among these, the CTX-M enzyme is recognized as the most dominant Extended-Spectrum β -Lactamase (ESBL) variant with the broadest global distribution. Both SHV (Sulfhydryl Variable) and CTX-M (Cefotaximase-Munich) genes encode these enzymes, which function by hydrolyzing the β -lactam ring to neutralize the antibiotic. Notably, this enzymatic inactivation represents a distinct resistance mechanism, functioning independently of membrane transport proteins or efflux pumps (Bush K, Bradford PA. 2020).

Bacterial infection and ischemia are the leading causes of amputation in diabetic ulcers; molecular systematics methods can accurately identify microorganisms. DNA multiplex protocols that analyze multiple genes will greatly improve genetic screening, serving as an early detection tool for health outcomes in diabetic patients with ulcer complications. Multiplex PCR is an effective technique for quickly detecting SHV, CTX-M, and *mecA* genes, which can benefit infection control efforts and help reduce morbidity and mortality among diabetic patients.

In Indonesia, research on antibiotic resistance genes in bacteria infecting diabetic ulcers remains limited, as most previous studies have focused on bacterial patterns and phenotypic antimicrobial resistance profiles (Akash et al., 2020; Salim et al., 2020). These conventional testing limitations have left genetic-level resistance mechanisms poorly mapped, particularly in the Mojokerto region. This study provides a specific contribution by performing molecular detection of SHV, CTX-M, and *mecA* genes using multiplex PCR—an approach not previously reported for patients at Wahidin Hospital, Mojokerto. Therefore, this study aims to identify the bacterial species and the presence of resistance genes (SHV, CTX-M, and *mecA*) in isolates from diabetic ulcer patients at Wahidin Hospital, Mojokerto.

MATERIALS AND METHODS

This was a descriptive observational study with a descriptive aimed at identifying bacterial profiles and detecting the presence of resistance genes in bacterial isolates

obtained from diabetic foot ulcers (DFU) of patients at RSUD Dr. Wahidin Mojokerto. Bacterial culture and identification genes were conducted at the Microbiology and Biotechnology Laboratory of Anwar Medika University Sidoarjo over three months, from August to November 2024. Inclusion criteria: DM patients diagnosed with diabetic ulcer complications, male and female genders, and samples taken from both inpatient and outpatient rooms. A total of 32 samples were obtained.

Sampling of Diabetic Ulcers

Sampling of diabetic ulcers was conducted using the sterile swab method, a clinical standard preferred for its ease of application and non-invasive nature (Travis et al., 2020). Specimens were collected from purulent exudate containing inflammatory proteins, leukocytes, interstitial fluid, and cellular debris.

The procedure commenced with obtaining informed consent and recording patient identification. The ulcer swab was performed using a zigzag technique across the wound bed, rotating the swab between the fingers while avoiding contact with the wound edges. This technique ensures a uniform distribution of microorganisms and sufficient biomass for subsequent inoculation on selective media (Idris et al., 2020). Following collection, the sterile swabs were immersed in screw-cap tubes containing Nutrient Broth (NB) as a transport medium. The samples were then transported to the Biotechnology Laboratory in an ice box to maintain specimen viability.

Culture and Identification of Bacteria

Sterile swab samples were cultured on Blood Agar Plate (BAP), MacConkey agar, and Eosin Methylene Blue (EMB) agar as general, selective, and differential media for the initial identification of Gram-positive and Gram-negative bacteria. As these media are not specific to particular bacterial species, observations of colony morphology were used only as a preliminary screening step. A total of 32 diabetic ulcer swab samples were recultured on these media and incubated at 37°C for 24–48 hours. Colony growth on BAP showed discrete, small, round, smooth colonies that were white to moderately pigmented, with some exhibiting whitish-yellow pigmentation. After 48 hours of incubation, clear zones surrounding the colonies were observed, indicating possible hemolytic activity, a characteristic commonly associated with *Staphylococcus* spp (Mita Zuliana et al., 2023). Colony growth on Mac Conkey and Eosin-Methylene Blue media showed that *E. coli* was characterized by small, sticky red colonies, while *Klebsiella pneumoniae* was characterized by large, red, and semi-mucilaginous colonies (Rizky et al., 2021).

To enhance the accuracy and validity of bacterial identification, all isolates were subjected to confirmatory testing. *Staphylococcus aureus* was identified using molecular methods targeting the *nuc* gene as a specific marker. In contrast, *Escherichia coli* and *Klebsiella pneumoniae* were identified using conventional methods, including cultivation on Tryptic Soy Agar (TSA) followed by a series of biochemical tests (Sugireng & Rosdarni, 2021).

Multiplex gene amplification

The initial stages of Multiplex gen amplification are DNA isolation and extraction using the thermal cycler dry boil method at 99 °C for 5 minutes, one ose suspended in 0.1 ml sterile distilled water, and then centrifuged at 6000 rpm for 10 minutes 23. The mixture for the PCR reaction with a volume of 25 µl consisted of PCR mastermix 12.5 µl (0.5 U Taq polymerase, 0.2 mM dNTP, 1.5 mM MgCl₂, and 1x buffer), 1.5 µl D.W, 1 µl for each primer, and 5 µl template DNA. The PCR temperature used was an initial denaturation at 95 °C for 7 minutes, followed by 35 cycles of 95 °C for 30 seconds (denaturation), 53 °C for 40 seconds (annealing), and 72 °C for 1 minute (extension),

and a final extension at 72 °C for 10 minutes. PCR products were visualised in 1.5% agarose gel and then electrophoresed.

Table 1. Primer Spesifik SHV, CTX-M, MeCA

No	Gene	Sekuens	Amplicon length
1	SHV	F 5"GGTTATGCGTTATTCGCC3" R 5"TTAGGTTGCCAGTGCTC3".	593 bp
2	CTX-M	F 5'-ATGTGCAYACCAGTAARGT-3' R 5'-TGGGTRAARTARGTSACCAGA-3'	876 bp
3	mecA	F 5'-AAAATCGATGGTAAAGGTTGGC- 3' R 5'-AGTTCTGCAGTACCGGATTTGC-3'	533 bp

Approval for this study was obtained from Ethics Comission of KEPK RSUD Dr Wahidin Sudiro Husodo Mojokerto, No. 41/KEPK-RSWH/EA/2024.

RESULTS AND DISCUSSION

Results Identification of Bacteria

Bacterial isolation and identification of diabetic ulcer samples yielded a total of 32 isolates from outpatients and inpatients. The results are shown in (Table 2).

Table 2. Bacterial Pattern of Diabetic Ulcer Isolates

	Bacterial species	Quantity	%
1	<i>Staphylococcus Aureus</i>	20	63%
2	<i>Escherichia coli</i>	9	28%
3	<i>Klebsiella pneumoniae</i>	3	9%

Bacterial identification in diabetic ulcer swab samples at Wahidin Regional Hospital, Mojokerto, showed that the most common bacteria were *Staphylococcus aureus* (63%). This finding aligns with research conducted (Mita Zuliana et al., 2023), Gram-positive infection-causing bacteria were identified in wound samples from 40 patients, including 9 *Staphylococcus aureus* samples and 9 *Staphylococcus epidermidis* samples, out of a total of 22 other samples. A 2019 study found that the most common bacterial species found in diabetic ulcer pus were *Klebsiella pneumoniae* (40%) and *Staphylococcus aureus* (10%). (Pratama Putri et al., 2025) explained that bacterial culture results showed that gram-negative bacteria, *Klebsiella pneumoniae* (17.9%), *Escherichia coli* (16.5%), and *Acinetobacter baumannii* (14.7%) dominated, while gram-positive bacteria dominated, with the most common cause being *Staphylococcus aureus* (16.5%).

Elevated blood glucose levels in patients with DM significantly increase the risk of complications, including wound infection and subsequent delayed healing. DM patients are inherently susceptible to secondary infections due to their compromised immune system. Furthermore, local factors such as poor wound hygiene, contact with unclean or moist environments, and improper care can also precipitate secondary infections (Salim et al., 2020). Several virulence factors (VFs) and toxins produced by *Staphylococcus aureus* during infection have been well-characterized. In Diabetic Foot Infections (DFI), *S. aureus* pathogenicity is strongly influenced by these VFs, particularly secreted toxins, which play crucial roles in bacterial colonization, persistence, and evasion of the host immune system (Hussein et al., 2024). Specifically, *S. aureus* contributes to diabetic ulcers through various virulence factors, including toxins (Lienard et al., 2021). *Escherichia coli*, a Gram-negative bacterium

that causes skin and soft tissue infections (SSTIs), has been isolated from ulcers in patients with diabetes mellitus (DM).

Results Multiplex gene amplification

Electrophoresis results showed successful DNA amplification from 32 bacterial isolates consisting of *Staphylococcus aureus* (S), *Escherichia coli* (E), and *Klebsiella pneumoniae* (KP). Detection of resistance coding genes in the 32 isolates showed that the SHV gene was detected in 3% (1 isolate) of *S. aureus* isolates. The CTX-M gene was found in *S. aureus* and *E. coli* isolates with a percentage of 19% (6 isolates), while the *mecA* gene was detected in *S. aureus* (4 isolates) and *E. coli* (3 isolates) with a percentage of 22% total of 7 isolates. The combination of the SHV and *mecA* genes was found in *S. aureus* isolates with a percentage of 3% (1 isolate), and the combination of the *MecA* and CTX-M genes was also detected in *S. aureus* with a percentage of 3% (1 isolate). Overall, of the 32 isolates tested, as many as 50% of the isolates contained genes coding for resistance and/or virulence.

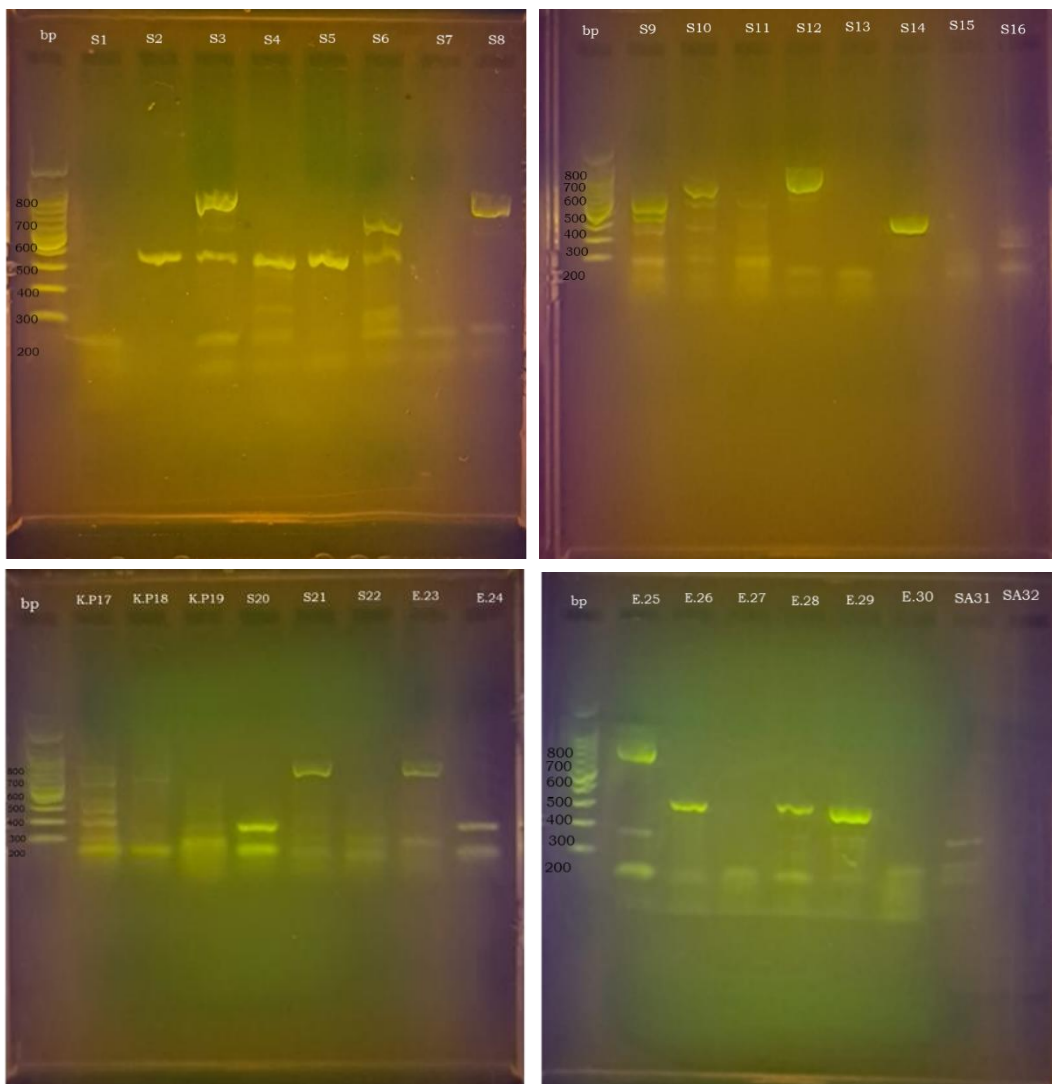


Figure 7. Electrophoresis profiles of multiplex PCR amplification from 32 bacterial isolates obtained from diabetic ulcer swab samples in four separate running gels. Amplification bands corresponding to *mecA* (533 bp), SHV (593 bp), and CTX-M (876 bp) genes were observed in several isolates.

Table 3. Result Multiplex Gene Amplification

No	Sample Number	Band Size (bp)	Multiplex Gene Amplification Results
1	S2, S4 and S5, S14	533	Mec-A
2	E26, E28, and E29	533	Mec-A
3	S8, S10, S12, S21, E23 and E25	876	CTX-M
4	S3	533 dan 876	CTX-M & MecA
5	S6	533 dan 593	MecA & SHV
6	S9	593	SHV

Multiplex PCR enabled the simultaneous detection of multiple resistance-associated genes in a single reaction using several primer pairs targeting different DNA sequences (Naully & Septriliyana, 2022). Samples S2, S4, S5, and S14 produced amplification bands at 533 bp, indicating the presence of the *mecA* gene in isolates identified as *Staphylococcus aureus* from diabetic ulcer swabs. Similarly, samples E26, E28, and E29 also showed amplification bands at 533 bp. However, the detection of the *mecA* gene in isolates identified as *Escherichia coli* is biologically uncommon and may indicate possible isolate misidentification, contamination during sample processing, or non-specific amplification during multiplex PCR analysis (Humaryanto et al., 2023).

Therefore, these findings should be interpreted cautiously and require further confirmation using standardized bacterial identification methods and singleplex PCR assays with species-specific targets. In addition, sequencing analysis is recommended to validate the specificity of the amplified products. The detection of ESBL-associated genes (CTX-M and SHV) in *Staphylococcus aureus* and the *mecA* gene in *Escherichia coli* does not correspond with the typical distribution of these resistance determinants in clinical isolates. This discrepancy may be associated with cross-contamination during DNA extraction or PCR preparation, primer cross-reactivity, or technical limitations inherent to multiplex PCR assays. Previous studies have reported that improper specimen collection and handling procedures may increase the risk of contamination and affect the accuracy of molecular detection (Humaryanto et al., 2023). Therefore, strict sampling procedures and molecular validation are essential to minimize false-positive amplification and improve the reliability of resistance gene detection.

Staphylococcus aureus is one of the most common causes of infection in diabetic patients. This microorganism plays an important role in this scenario as it causes a variety of infections, ranging from superficial infections to serious and potentially fatal systemic infections. Infections caused by *methicillin-resistant Staphylococcus aureus* (MRSA) are associated with higher mortality rates than those caused by *methicillin-susceptible Staphylococcus aureus* (MSSA) (Teixeira et al., 2021). Several studies have reported high levels of antimicrobial resistance in MRSA isolated from diabetic patients. 15 of 112 *Staphylococcus aureus* isolates carried the methicillin-resistant *mecA* gene (Teixeira et al., 2021; Hussein et al., 2024). *MecA* genotyping by PCR remains the main recommendation, although detection of MRSA phenotypes by disc diffusion still does not provide accurate results and cannot be performed routinely (Rafif Khairullah et al., 2022).

Electrophoresis of samples numbered S8, S10, S12, S21, E23, and E25 showed a band of 876 bp, indicating the presence of the *ctx-m* gene in *Staphylococcus aureus* and *E. coli*. CTX-M is a type of extended-spectrum β -lactamase (ESBL) that

can hydrolyse antibiotics and cause resistance. Plasmids in bacteria contain genes that can code for ESBL enzymes. These enzymes are TEM, SHV, CTX-M, and OXA. However, one that is commonly found is the CTX-M enzyme. ESBL is commonly found in bacteria from the Enterobacteriaceae family, including *Escherichia coli*, and these bacteria can hydrolyse penicillin antibiotics (Pratama et al., 2019).

The electrophoresis of the sample *Staphylococcus aureus* (S3) showed two bands of 533 bp and 876 bp, indicating the presence of *mec-A* and *ctx-m* genes. Sample S6 showed bands of 533 bp and 593 bp, indicating that the *mecA* and *shv* genes were identified in the sample. Sample S9 showed a band of 593 bp and was identified as the *shv* gene. Previous studies on the proportion of ESBL-producing bacteria throughout Japan showed that *Escherichia coli* accounted for 14.0%, and *Klebsiella oxytoca* for 3.3%, *Klebsiella pneumoniae* (3.1%), and 15.9% of *Proteus mirabilis* were ESBL producers. All these isolates belonged to the CTX-M group (Shibasaki et al., 2016). In another study conducted in Japan, 36 (33.6%) of 107 *E. coli* strains tested carried only CTX-M type genes, and 62 (57.9%) carried both CTX-M type and TEM type genes or SHV. Only 9 strains (8.4%) carried TEM and/or SHV type genes without CTX-M type genes (Abe et al., 2019). The sulfhydryl variable (SHV) gene is one of the most frequently identified Extended-Spectrum Beta-Lactamase (ESBL) coding genes. Research results related to the development of a multiplex PCR system to detect the multiplex CTX-M, *bla*TEM, and OXA-1 genes, through testing on 203 clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli*, the use of specific primers optimized from singleplex to multiplex reactions successfully demonstrated 100% sensitivity and specificity. Accuracy in determining this β -lactamase subtype has also been confirmed through sequencing of PCR products (Ghenea et al., 2022).

This study is limited by a relatively small sample size, the use of swab sampling, which may not adequately represent deep tissue infections, and the absence of phenotypic antibiotic susceptibility testing to support the genotypic findings. Furthermore, the lack of standardized bacterial identification methods across isolates and the absence of confirmatory approaches such as singleplex PCR or sequencing may affect the accuracy of the results. The detection of resistance genes in atypical bacterial hosts, such as *Staphylococcus aureus* and *Escherichia coli*, should also be acknowledged as a potential methodological limitation. Therefore, future studies should consider larger sample sizes, more representative sampling techniques (e.g., tissue biopsy), integration of phenotypic and genotypic resistance testing, and rigorous validation of PCR results using appropriate controls and sequencing methods to strengthen the reliability of the findings.

CONCLUSION

The identification of 32 diabetic ulcer swab samples collected from inpatients and outpatients with diabetes at Wahidin Hospital, Mojokerto, showed that the most common bacterial populations were *Staphylococcus aureus* (63%), *Escherichia coli* (28%), and *Klebsiella pneumoniae* (9%). Furthermore, multiplex gene detection encoding antibiotic resistance in the identified isolates revealed the following distribution: SHV genes 4%, CTX-M 19%, *mecA* 22%, multi-gene SHV & *mecA* 3%, and multi-gene CTM-X & *mecA* 3%. These results confirm the successful use of multiplex PCR to detect genes encoding antibiotic resistance in bacteria infecting ulcers in patients with diabetes.

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CONFLICT OF INTEREST

There is no conflict of interest in this research.

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