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## Identification of bacterial species isolated from diabetic foot ulcer in Iraq

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### ABSTRACT

The main goal of the study is the isolation and diagnosis of bacterial species causing diabetic foot. Diabetic foot ulcers are complex infections arising from a combination of microbial and host factors. DFUs typically harbor a diverse array of microorganisms, encompassing aerobic and anaerobic bacteria, fungi, and occasionally viruses. Frequent bacterial culprits in diabetic foot ulcers are *Staphylococcus* spp, and a variety of gram-negative rods. In particular swabs were taken from diabetic individuals and cultivated in conditions that were conducive to the growth of bacteria. Following that, they underwent diagnosis using the VITEK2 system in the laboratory. The VITEK 2 System is an automated microbiology system used primarily for the identification and antibiotic susceptibility testing of bacteria 16SrRNA gene (plays a crucial role in the process of translating genetic information from mRNA into proteins). The gene encoding 16SrRNA is commonly used in molecular biology for various applications, particularly in the identification and classification of microorganisms), isolates saved for the additional examination of gene sequencing to determine the type of bacteria. A total of 50 swabs were positive for bacterial culture, the percentage of *Staphylococcus* spp, was 30% compared to *Enterobacteriaceae* spp, was 12% while other bacterial causes were 58% of the total percentage 100% included (*Achromobacter*, *Wautersiella falsenii*, *Proteus alimenterum*). The current article concludes with the identification of bacterial species in diabetic foot ulcers, phenotypic and laboratory diagnosis, and selecting pure isolates for diagnosis confirmed by 16S rRNA gene sequencing.

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### Introduction

Diabetes mellitus (DM) is a condition with consequences that are growing at a concerning rate (Kateel et al. 2018). A common consequence of diabetes, (DFU) are characterized by pain or soreness that typically affects the bottom of the foot, if left untreated, these ulcers can become more serious and challenging to treat (Yazdanpanah et al. 2015).

Diabetic foot ulcers (DFUs) show individuals of the ultimate frequent complexities of diabetes mellitus, particularly in underdeveloped countries, and can cause

restriction and melancholy. Approximately 25% of diabetics will evolve bottom ulcers throughout their period (Thanganadar Appapalam et al. 2021).

According to the International Working Group on the Diabetic Foot (IWGDF), a diabetic basis of an object abscess is an adequate-girth wound penetrating through the especially of animate being (the open ocean vascular and photomontage-innate intelligence interlining of the skin) located beneath the bone in a diabetic patient (Jalilian et al. 2020). Extreme age, heat, high body mass index, glucose, type of diabetes, and other problems in

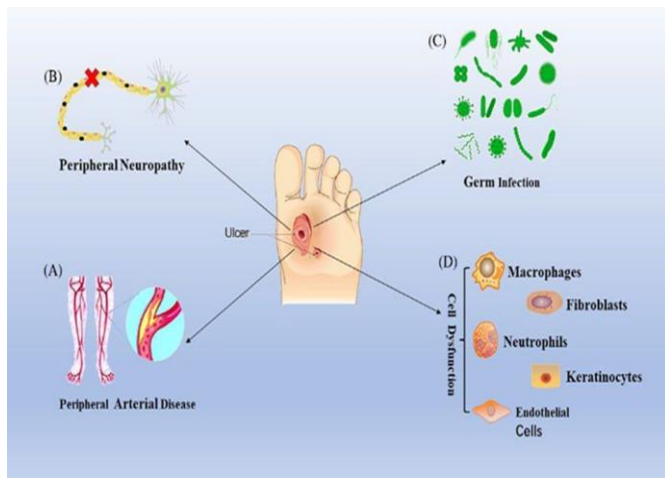
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settings are components that appear in several research studies. (Moeini et al. 2017; Madmoli et al. 2019).

The pathogenesis of DFU involves a multifactorial interplay of various systemic and local factors. These factors contribute to the development, progression, and chronicity of DFU, such as of the key components; peripheral neuropathy, peripheral vascular disease (PVD), biomechanical abnormalities, hyperglycemia, impaired wound healing, and infection (Qi et al. 2018).

Diabetic foot neuropathy is roughly linked with age, disease course, and consideration of diabetes control. Among diabetes problems, the clinical disease prompted by an injury to the minor and unrestrained politically fearful systems because of the unstable metabolic environment in diabetes, the healing alterations in the nervous system have close connection with the fundamental and functional disorders of nerve containers (Volmer-Thole & Lobmann, 2016). Peripheral vascular disease (PVD), also known as minor channel ailment (PAD), is a low snag in sufferers of diabetes and plays a significant impact on the development and progression of diabetic foot ulcers (Cassar et al. 2010). The following is a reason of the relationship between PVD and DFUs, as shown in **figure 1** according to Macdonald et al. (2021).



**Fig 1** Four major elements of diabetic foot ulcer formation: peripheral vascular disease, peripheral neuropathy, and bacterial infection (Macdonald et al, 2021).

**Accelerated Atherosclerosis:** Diabetes steps up the process of atherosclerosis, ordering plaques amplified in the arterial obstruction, superior to reduced and numb channels. This reduces clot flow to the limits.

**Endothelial Dysfunction:** High levels of glucose in blood levels damage the endothelium, impairing allure function and promoting atherosclerotic memorial establishment.

**Inflammation:** Chronic inferior inflammation associated with diabetes contributes to the progress of atherosclerosis.

### Bacterial diversity of diabetic foot ulcer

The infection is commonly complicated to (DFU) beginning with an open wound on the foot caused by injury, ischemia, or foot, the injured invulnerable construction, injured peripheral blood classification, neuropathy, and peripheral vasculopathy cut down (Church et al. 2020).

The development of foot ulcers, particularly in people with diabetes, is affected by multiple aspects of wound microbiology, and these include microbial load, bacterial biodiversity, and the life cycle of hazardous species (Kurup & Ansari, 2019). The bacteriology of (DFUs) is a key feature in understanding their pathogenesis, directing situations, and developing patient consequences (Anandi et al. 2004).

The microbial environment of DFUs is generally polymicrobial and involves a mix of aerobic and anaerobic bacteria, which can confuse pathogenic management. *Staphylococcus aureus* is often isolated from (DFUs) due to its virulence and biofilm-forming ability, which shields it from the immune system. It is one of the most common organisms associated with DFUs and a leading cause of diabetic foot infections (Lee et al. 2023). *Staphylococcus aureus* is known to infect bone and survive intercellularly within several bone cell types, including osteoblasts and osteocytes, and is frequently responsible for osteomyelitis, a common DFU complication.

*Staphylococcus hemolyticus*, a member of the coagulase-negative staphylococci (CoNS) group, is known to be involved in skin wounds and (DFUs). It can colonize chronic wounds, including DFUs. Although it is less virulent than *S. aureus*, *S. hemolyticus* can still contribute to infections, particularly in immunocompromised individuals, such as those with diabetes and despite being generally less virulent than *S. aureus*, *S. hemolyticus* secretes enzymes and toxins that can damage tissue and hinder healing. In addition to another type, *Staphylococcus sciuri* is commonly present in the environment and on the skin of animals and humans, although it is generally less virulent than *Staphylococcus aureus*, it can also function as an opportunistic pathogen, particularly in immunocompromised individuals or those with chronic wounds like (DFUs) (McManus et al. 2020).

In DFUs, *Staphylococcus sciuri* can play a significant role as an opportunistic pathogen, leading to persistent infections and impeding the healing process (Al-Muhanna et al. 2020). The Enterobacteriaceae family

includes bacteria such as *Escherichia coli*, *Klebsiella* spp., *Proteus* spp., especially *Proteus alimenterum* were present in polymicrobial infections of (DFUs) and these bacteria can increase the complexity and severity of the infection, also can produce a range of virulence factors, such as urease, proteases, and hemolysins, which can damage tissue, hinder healing, and facilitate the spread of the infection (Shahi & Kumar, 2016). *Achromobacter* species, though not as frequently linked to diabetic foot ulcers (DFUs) as other bacteria, can still contribute to skin wounds and DFUs, are opportunistic pathogens found in chronic wounds, including DFUs, while less prevalent, they add to the infection's complexity. These bacteria have various virulence factors that allow them to colonize and persist in wounds, such as the ability to adhere to surfaces, resist phagocytosis, and secrete tissue-degrading enzymes (Ferhaoui et al. 2023). *Wautersiella falsenii*, though relatively rare and infrequently encountered, is an opportunistic pathogen that can contribute to infections, such as (DFUs). Its occurrence in DFUs may complicate treatment due to its potential for antibiotic resistance and the inherent difficulties in managing chronic wounds (Abdulrazak et al. 2005). These bacteria are frequently present in polymicrobial infections and can lead to severe infections, particularly in patients with compromised immune systems.

### Materials and Methods Sample collection

The study was conducted from November 2023 to March 2024; clinical samples were collected from 50 patients with diabetic foot ulcers were conducted from Marjan Teaching Hospital (Babylon, Iraq) and Al-Kafeel Specialized Hospital (Karbala, Iraq).

### Bacterial identification

Samples from diabetic wounds are collected using swab media to transfer them from the hospital to the laboratory. Once collected, the samples were cultured on specific media that were prepared according to the manufacturer's instructions. The isolated colonies formed were streaked onto Mannitol Salt Agar, a selective and differential medium mainly used for isolating and identifying *Staphylococcus* spp. MacConkey agar, another selective and differential culture medium, is designed for isolating and differentiating Gram-negative enteric bacteria through their lactose fermentation ability. Eosin Methylene Blue agar is primarily used to isolate and distinguish Gram-negative enteric bacteria and incubated at 37°C for 24 hours.

### Bacterial diagnosis

This involves identifying colonies based on their morphology, microscopic examination, biochemical tests, and confirmation using the VITEK2 system.

### Molecular identification of bacterial species using 16SrRNA gene DNA isolation and extraction

Bacterial genomic DNA was extracted from the species using Genomic DNA Kit (Promega, the USA), following the manufacturer's instructions, which included glass bead beating for cell wall disruption. The extracted DNA served as a template for PCR amplification by using a universal bacterial primer and amplification by PCR in the optimum condition and components of PCR as shown in Tables 1-3 (supplementary).

### Amplification of DNA by PCR

The components required for amplification of DNA by PCR as shown in Table 3 (supplementary materials).

### Result and discussion

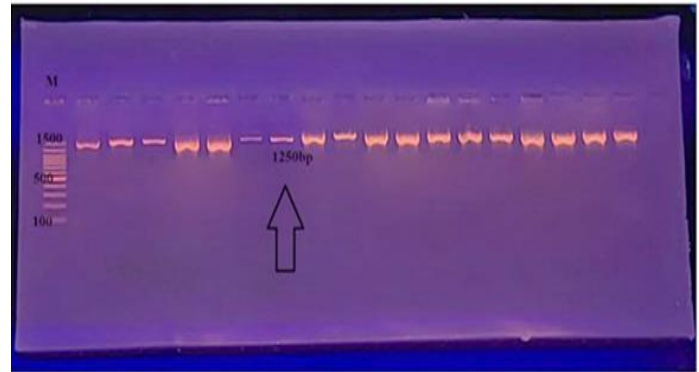
The mean relative abundance of bacteria from genera with recognized and potential pathogens was frequently greater in swab samples such as *Staphylococcaceae* spp, *Achromobacter* spp., *Enterobacteriaceae* spp., and the percentage of bacterial species developed in this investigation was as follows: *Staphylococcus* spp. (26%), *Enterobacteriaceae* spp. (12%), and the remaining 58% were split among the bacterial species with the lowest proportion, *K pneumoniae*, *Morgonella* spp, *Wautersiella falsenii* and *Achromobacter* spp, as shown in Table 4 (supplementary materials). 16S rRNA sequencing technology detected significantly more genera of bacteria and polymicrobials in diabetic foot infection. Our findings highlight 16S rRNA sequencing as a viable approach for the bacteriological diagnosis of DFU, using 16S rRNA gene sequencing, identifying pathogenic bacteria in DFU is fast (2.3-7.3h running time, total process <24h) and leads to a rapid diagnosis of the bacterial community. The selected isolates in our study as shown in Table 5 (supplementary materials) by comparison with previous studies, it is clear that, bacteria causes most diabetes, bacteria commonly isolated from wounds include *S. aureus*, gram-negative organisms, and some anaerobic bacteria. The most frequently isolated organisms are *Staphylococcus aureus* (38.4%), *Proteus mirabilis* (4%) and *Enterococcus* species (Thanganadar Appapalam et al. 2021). Gram-negative organisms obtained were *Escherichia coli*, *Proteus* spp, *Enterobacter* spp, *K pneumoniae*, *Morgonella* spp (Travis et al. 2020) Similar to their study, *Staphylococcus* spp. it is most common in swabs and *Enterococci* spp isolated from diabetic foot ulcers (Church et al. 2020).

Another study added the investigation also showed that the molecular profiles of some bacterial species like *Corynebacterium striatum*, *Proteus mirabilis*, or *Morganella morganii* were affected by the composition of the culture medium, which led to variations in the accuracy of the identification (Złoch et al. 2021). Another study noted that, out of 180 samples, the most frequently isolated pathogenic bacteria, identified through differential media, morphological, and biochemical tests, were *S. aureus* at 98 instances (54%) and *E. coli* at 75 instances (41.6%) (Church et al. 2020).

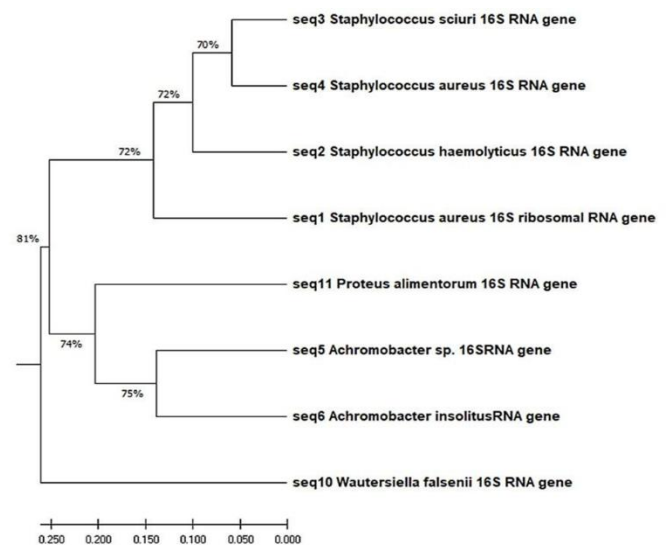
*S. epidermidis*, at 20 instances (11.1%), was the least common among all isolates it was *S. sciuri* is typically regarded as a commensal organism, it has been associated with various infections, especially in individuals with compromised immune systems and it has been identified in instances of bacteremia and wound infections., also utilized 16S rRNA sequencing to confirm bacterial identification (Salah et al. 2022) were also obtained the same result in our study , *staphylococcus* spp are the most prevalent bacteria found in diabetic wounds, one hundred bacterial isolates were recovered from diabetic foot ulcers, belonging to five genera: *Staphylococcus* sp., *Klebsiella* sp, *E.coli*, and *Proteus* spp, *S. hemolyticus* infections caused by in (DFUs) can result in chronic wounds, a higher risk of amputation, and extended hospital stays. However, the bacterium's capacity to endure in the wound environment and resist treatment contributes to adverse clinical outcomes and elevated healthcare costs, phenotypic analysis confirmed these findings, which were further supported by phylogenetic analysis (Vanechoutte et al. 2011).

A substantial evidence resented by a previous study it is consistent with our study that included *Wautersiella falsenii* has been identified in various clinical samples, including wound infections, its occurrence in (DFUs) is clinically significant as it can affect the progression and treatment of these chronic wounds, although *W. falsenii* is not frequently encountered, it may contribute to the polymicrobial nature of DFU infections, potentially complicating the healing process through its interactions with other microorganisms, *W. falsenii* can aggravate chronic inflammation and impede the healing process . *Achromobacter* species, typically present in water and soil, are recognized for causing infections in humans, particularly in those with compromised immune systems, however, another author confirmed their occurrence in (DFUs) complicates infection management due to their pathogenic potential (Jnana et al. 2020). PCR is a widely utilized method for amplifying specific DNA sequences, enabling the production of millions of copies from a minimal initial DNA sample. Figure (2) shows bands were fractionated by electrophoresis on a 1.5% agarose

gel, and visualized under UV. Phylogenetic analysis of bacteria from (DFUs) is vital for comprehending the microbial diversity and potential pathogenic interactions in these infections, also the microbiome diversity the assessment of the overall microbiome diversity is crucial for understanding its impact on wound healing and infection, result of this study is shown in Figures 3 and Table 5 (supplementary materials).



**Fig. 2.** Agarose gel electrophoresis for gene. Electrophoreses separated bands on 1.5% agarose gel and observed under UV light. Lane M (100bp ladder)



**Fig. 3.** Phylogenetic tree analysis was conducted based on the partial sequences of the 16S ribosomal RNA from various strains. Eleven isolates from Iraq, designated as IQD-No.1 to IQD-No.8, were used for genetic species typing analysis. The phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in MEGA version 6.0.

## Conclusion

All samples were cultured on three distinct types of media: nutrient agar, MacConkey agar, and mannitol salt agar, to identify both gram-negative and gram-positive bacteria. Biochemical, morphological, and molecular *16S rRNA* gene investigations were utilized to characterize the bacterial species. Molecular identification of isolated species was carried out by amplifying the *16S rRNA* gene using primers. The sequencing data revealed that the phylogenetic tree of bacterial species from this study clustered together and with reference sequences, indicating high similarity based on *16S rRNA*. These sequencing results further confirm the bacterial identification through sequence BLAST analysis. The most commonly isolated organisms from DFUs were *S. aureus* and *E. coli*. The lowest among all the isolates were *S. epidermidis*, *Achromobacter*, *Wautersiella falsenii* and *Proteus alimenterum*, *S. hemolyticus*, *S. epidermidis*. Enhancing general awareness programs can halt the spread of infections and, crucially, reduce the risk of lower extremity amputations through multimodal strategies, advanced diagnostic methods, proper use of antibiotics, surgical procedures, and regular foot assessments. The proper identification and treatment of the bacteria can prevent the spread of infection to deeper tissues, bones (osteomyelitis), or the bloodstream (sepsis).

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## Conflict of interest

The authors have no conflicts of conflict to declare.

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## Ethical approval

The ethical committee of the department of parenting, College of Women's Sciences at Babylon University authorized the study. Patients were informed about participation in the study and received it orally. According to the document, the local ethics committee reviewed and approved the study protocol, study data, and consent, form number 26 / June 2023.

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