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# Molecular Phylogenetic Demonstration of *Staphylococcus Aureus* in Diabetic Foot Ulcers

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## Abstract

**Background**: In Iraq, the high incidence and recurrence rates of diabetic foot ulcers (DFUs) underscore the need for a multifaceted approach to prevention and management.

**Aim**: Direct molecularly detection the prevalence of *S. aureus* among the DFUs patients using of molecular assay; and then, nucleotide sequencing of some local *S. aureus* isolates to be documented in the NCBI and identify its association with the GenBank-BLAST *S. aureus* isolates. Distribution of infection among the age and sex of study population in addition to type of medication(s) were aimed, also.

**Materials and methods**: After disinfecting of skin surrounding the ulcer, pus samples were collected from 73 DFU patients using sterile swabs that examined molecularly by the PCR assay. DNAs of some positive samples were sequenced and analysed phylogenetically.

**Results**: Targeting the *16S rRNA* gene, 20.55% of DFU swabs were positive reactivity to *S. aureus*. Concerning age, significant higher prevalence and risk of *S. aureus* were shown in patients aged ≥61 years old, and to less extent among 51- 60 years old when compared to other age groups; 41-50 and ≤40 years old. For sex, although no significant variation was seen among females and males, risk values revealed that males were at higher risk of *S. aureus* infection than females. Regarding the type of medication, DFU patients received injectable medication have significantly higher values of prevalence rate and risks than those of oral and mixed medications. Relation to phylogeny, the sequenced data of six local *S. aureus* strains were submitted in the NCBI database under specified names (DFU1-DFU6) and GenBank IDs (PQ318365.1- PQ318370.1). The findings of phylogentic tree analysis, homology sequence identity, and NCBI MSA viewer demonstrated that the local *S. aureus* strains were related to the NCBI-BLAST Iraqi isolate (LC576397.1) at an identity rate ranged from 98.65% to 99.79% and mutation/change at 0.02%.

**Conclusion**: This study confirms, for the first time in Iraq, the phylogenetic linkage of *S. aureus* isolated from DFU patients to the NCBI-GenBank *S. aureus* isolates. This study shows, also, significant differences in prevalence rates and risks of *S. aureus* among various age and sex groups as well as among DFU patients received different medications. Therefore, establishing the underlying causes of diabetes and developing effective interventions to prevent and manage its associated complications are crucial steps towards reducing the burden of this widespread disease. Additionally, the high incidence and recurrence rates of DFU remain underscore and need for a multifaceted approach to prevention and management.

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**Keywords**: Polymerase chain reaction (PCR), Sequencing analysis, Diabetes mellitus, National Centre For Biotechnology Information (NCBI), Iraq

## Introduction

*Staphylococcus aureus* is an opportunistic Gram-positive, facultative anaerobic coccus that is a common inhabitant of human microbiota, particularly in the nasal passages and on the skin (Ibraheim et al., 2023a). The ubiquitous existence and ability to produce a diverse array of virulence factors (toxins, enzymes, and cell surface proteins) enable it to evade host defenses and cause a wide range of infections that ranged from relatively mild skin and soft tissue infections to life-threatening conditions such as pneumonia, endocarditis, osteomyelitis, and sepsis (Park and Ronholm, 2021; Ibraheim et al., 2023b). Additionally, the bacterium has an ability to growing in a wide range of temperatures, pH, and sodium chloride concentrations and to survive dry and stressful environments which further contributes to cause food-related outbreaks (Alreshidi et al., 2020). In last decades, the emergence of antibiotic-resistant strains, such as methicillin-resistant *S. aureus*, has further exacerbated the clinical challenge posed by this pathogen, making it a significant public health concern (Abebe and Birhanu, 2023).

Diabetes mellitus is a chronic metabolic disorder characterized by elevated blood glucose levels due to inability of body to properly produce (type 1) or utilize (type 2) insulin, a hormone essential for regulating blood sugar (Al-Shaeli et al., 2022). The sustained hyperglycemia associated with diabetes can lead to a multitude of debilitating microvascular and macrovascular complications, affecting the cardiovascular system, kidneys, eyes, and peripheral nerves (Rangel et al., 2019; Mota et al., 2020). Diabetic foot ulcers (DFUs) are a significant complication of diabetes mellitus causing substantial morbidity, reduced quality of life, and increased healthcare costs (Edmonds et al., 2021). DFUs occur due to a complex interplay between several factors including peripheral neuropathy, peripheral arterial disease, and impaired wound healing (Davis et al., 2018). Neuropathy, leads to loss of sensation, can result in unnoticed trauma and the development of skin lesions (Pokhriyal et al., 2018). Peripheral arterial disease reduces blood flow to the lower extremities, impairing nutrient and oxygen delivery to the wound site, thereby hindering healing (Soyoye et al., 2021). Additionally, diabetes-induced alterations in the inflammatory response and impaired angiogenesis contribute to the delayed and dysfunctional healing characteristic of DFUs (Nirenjen et al., 2023). Hence, the prevention and early detection of these risk factors are crucial in reducing the incidence of DFUs (Lim et al., 2017). In addition, infection control can effectively manage DFUs since several infections lead to sepsis, gangrene, amputation, and even death (Pitocco et al., 2019).

In Iraq, few studies have investigated the prevalence of different microorganisms in DFUs individuals using of traditional techniques (Alkhudhairy and Al-Shammari, 2020; Qadir et al., 2020) or by the molecular PCR assay only (Hussein and Saleh, 2024). This study was performed to direct molecularly detection the prevalence of *S. aureus* among the DFUs patients using of PCR assay; and then, nucleotide sequencing of some local *S. aureus* isolates to be documented in the NCBI and identify its association with the GenBank-BLAST *S.*

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*aureus* isolates. Distribution of infection among the age and sex of study population in addition to type of medication(s) were aimed, also.

## Materials and methods

### *Ethical approval*

The final acceptance and approval was awarded by the Scientific Committee of Department of Basic Sciences in the College of Dentistry (University of Wasit, Wasit-Iraq). Samples were obtained post oral agreement of the DFUs patients.

### *Samples*

After disinfecting of the skin surrounding an ulcer, pus samples were collected from 73 DFU's patients using sterile swabs by a circular motion, kept into plastic containers, transported cooled to the laboratory and subjected directly for molecular processing. Data concerned to age and sex of study patients and type of medication(s) were recorded.

### *Molecular PCR assaying*

According to manufacturers' instructions of the Presto Mini gDNA Bacteria Kit (Geneaid, Taiwan), DNAs were extracted from the pus samples and examined by the Nanodrop System (Thermo Sientific, UK). Targeting the *16S rRNA* gene, one set of primers [(F: 5´- GGA ACT GAG ACA CGG TCC AG -3´) and R: (5´- GGG TCC CCG TCA ATT CCT TT -3´)] were designed for the current study based on the NCBI-GenBank *S. aureus* (PQ010750.1) isolate to prepare the Mastermix tubes at a final volume of 20µl using the GoTaq Green Master Mix Kit (Promega, Korea). For PCR reaction, the following conditions of the Thermal Cycler system (BioRad, USA) were followed: 1 cycle for initial denaturation (95°C / 5 minutes); 30 cycles for denaturation (95°C / 30 seconds), annealing (54°C / 30 seconds) and extension (72°C / 30 seconds); and 1 cycle for final extension (72°C / 7 minutes). Electrophoresis of PCR products in Agarose-gel (1.5%) stained with Ethidium Bromide was conducted at 80Am and 100Volt for 90 minutes, and the product size of positive samples was identified under the UV illuminator (Clinx Science, China) at approximately 616bp.

### *Phylogenetic analysis*

DNAs of some positive *S. aureus* samples were selected and sent to the Macrogen Company (Korea). The received sequence data were submitted initially in the NCBI-GenBank database to get the specific access numbers for the local *S. aureus* study isolates, and then subjected to the multiple sequence alignment analysis and phylogenetic tree analysis in the MEGA-11 Software to identify its identity to the NCBI-BLAST *S. aureus* isolates.

### *Statistical analysis*

The *t*-test, Odds ratio (OR), as well as Relative Risk (RR) in the GraphPad Prism Software (*version* 8.0.2) were served to evaluate significant differences between study values at $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$. The calculated OR were involved the value and 95%CI, while the calculation of RR was included the value, 95%CI, and NNT (Gharban, 2023).

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## Results

Targeting the *16S rRNA* gene, 20.55% (15/73) of study samples were showed a positive reactivity to *S. aureus* using the PCR assay (Figures 1, 2).

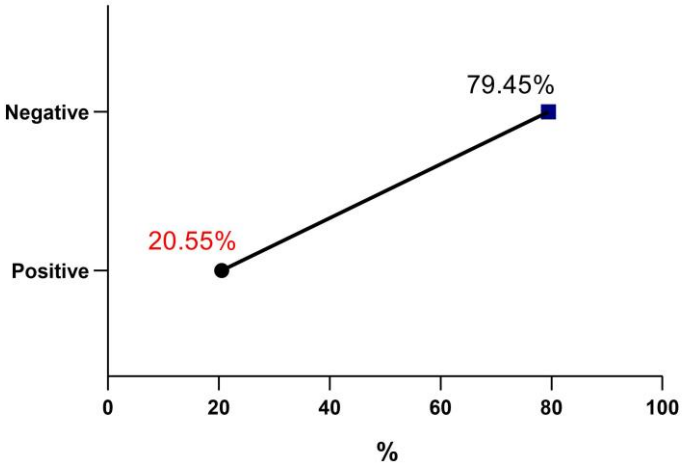


**Figure (1): Molecular prevalence rate (%) of *S. aureus* among the DFU study patients**

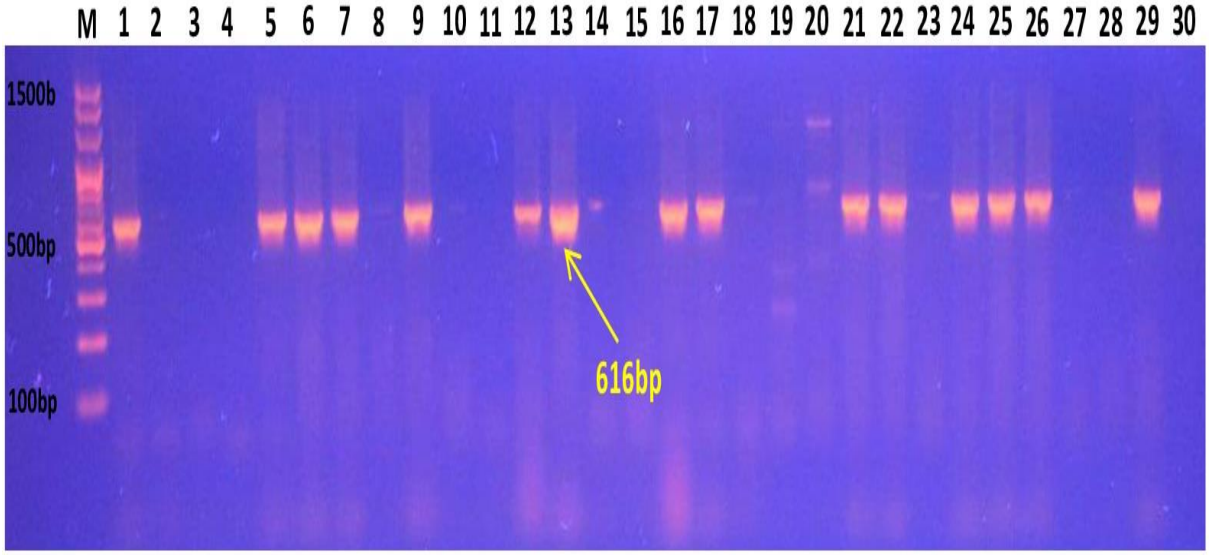


**Figure (2): Agarose-gel electrophoresis of PCR products at 80Am and 100Volt for 90 minutes**

Lane (M): Ladder marker (100-1500 bp)

Lanes (2, 3, 4, 8, 10, 11, 14, 15, 18, 19, 20, 23, 27, 28, and 30): Negative samples

Lanes (1, 5, 6, 7, 9, 12, 13, 16, 17, 21, 22, 24, 25, 26, and 29): Positive samples to *S. aureus* at 616bp

Concerning age of study population, significant higher values of prevalence rate, OD and RR of *S. aureus* were shown in ≥61 years old DFU patients (33.33%, 2.5556, and 1.7778, respectively) and to less extent in 51- 60 years old (23.53%, 1.4066, and 1.2117, respectively)

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when compared to other age groups; 41-50 years old (8.33%, 0.3052, and 0.4121, respectively) and ≤40 years old (0%, 0.2190, and 0.2581, respectively).

For sex, although no significant variation (p≤0.93) was seen in prevalence rate of *S. aureus* among females (20%) and males (20.59%), values of OR and RR revealed that DFU males (1.0370 and 1.0244, respectively) were at higher risk of *S. aureus* infection than females (0.9643 and 0.9762, respectively).

Regarding the type of medication, DFU patients received injectable medication have significantly higher values of prevalence rate (29.03%), OR (2.4545) and RR (1.8) than those of oral (12.5%, 0.4835 and 0.5983, respectively) and mixed (15.39%, 0.5950, and 0.7030, respectively) medications (Table 1).

**Table (1): Distribution of *S. aureus* infection among various risk factors**

| Factor | Group | Total No. | Positive [No. (%)] | OR | | RR | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Value | 95%CI | Value | 95%CI | NNT |
| Age (Year) | ≤ 40 | 9 | 0 (0%) | 0.2190 | 0.0121 to 3.9701 | 0.2581 | 0.0167 to 3.9903 | 6.957 Benefit |
| | 41-50 | 12 | 1 (8.33%) | 0.3052 | 0.0362 to 2.5742 | 0.4121 | 0.0591 to 2.8718 | 9.112 Benefit |
| | 51- 60 | 34 | 8 (23.53%) | 1.4066 | 0.4504 to 4.3927 | 1.2517 | 0.4968 to 3.1539 | 26.108 Harm |
| | ≥ 61 | 18 | 6 (33.33%) * | 2.5556 **** | 0.7600 to 8.5936 | 1.7778 **** | 0.7082 to 4.4625 | 9.143 Harm |
| ***p-value*** | | | *0.0118* | *0.0001* | - | *0.0001* | - | - |
| Sex | Female | 5 | 1 (20%) | 0.9643 | 0.0997 to 9.3227 | 0.9762 | 0.1532 to 6.2191 | 246 Benefit |
| | Male | 68 | 14 (20.59%) | 1.0370 *** | 0.1073 to 10.0261 | 1.0244 *** | 0.1608 to 6.5262 | 246 Harm |
| ***p-value*** | | | *0.093* | *0.0002* | - | *0.0003* | - | - |
| Medication | Oral | 16 | 2 (12.5%) | 0.4835 | 0.0971 to 2.4084 | 0.5983 | 0.1482 to 2.4158 | 13.404 Benefit |
| | Injection | 31 | 9 | 2.4545 | 0.7685 | 1.8 | 0.7004 | 10 |

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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | (29.03%) * | **** | to 7.8394 | **** | to 4.6262 | Harm |
| | Mixed | 26 | 4 (15.39%) | 0.5950 | 0.1686 to 2.1006 | 0.7030 | 0.2445 to 2.0213 | 17.755 Benefit |
| ***p-value*** | | | *0.0458* | *0.0001* | - | *0.0001* | - | - |

Relation to phylogeny, the sequenced data of six local *S. aureus* strains were submitted in the NCBI database under specified names (DFU1-DFU6) and GenBank IDs (PQ318365.1-PQ318370.1). The findings of phylogentic tree analysis, homology sequence identity, and NCBI MSA viewer demonstrated that the local *S. aureus* strains were related to the NCBI-BLAST Iraqi isolate (LC576397.1) at an identity rate ranged from 98.65% to 99.79% and mutation/change at 0.02% (Figures 3-5, Table 2).

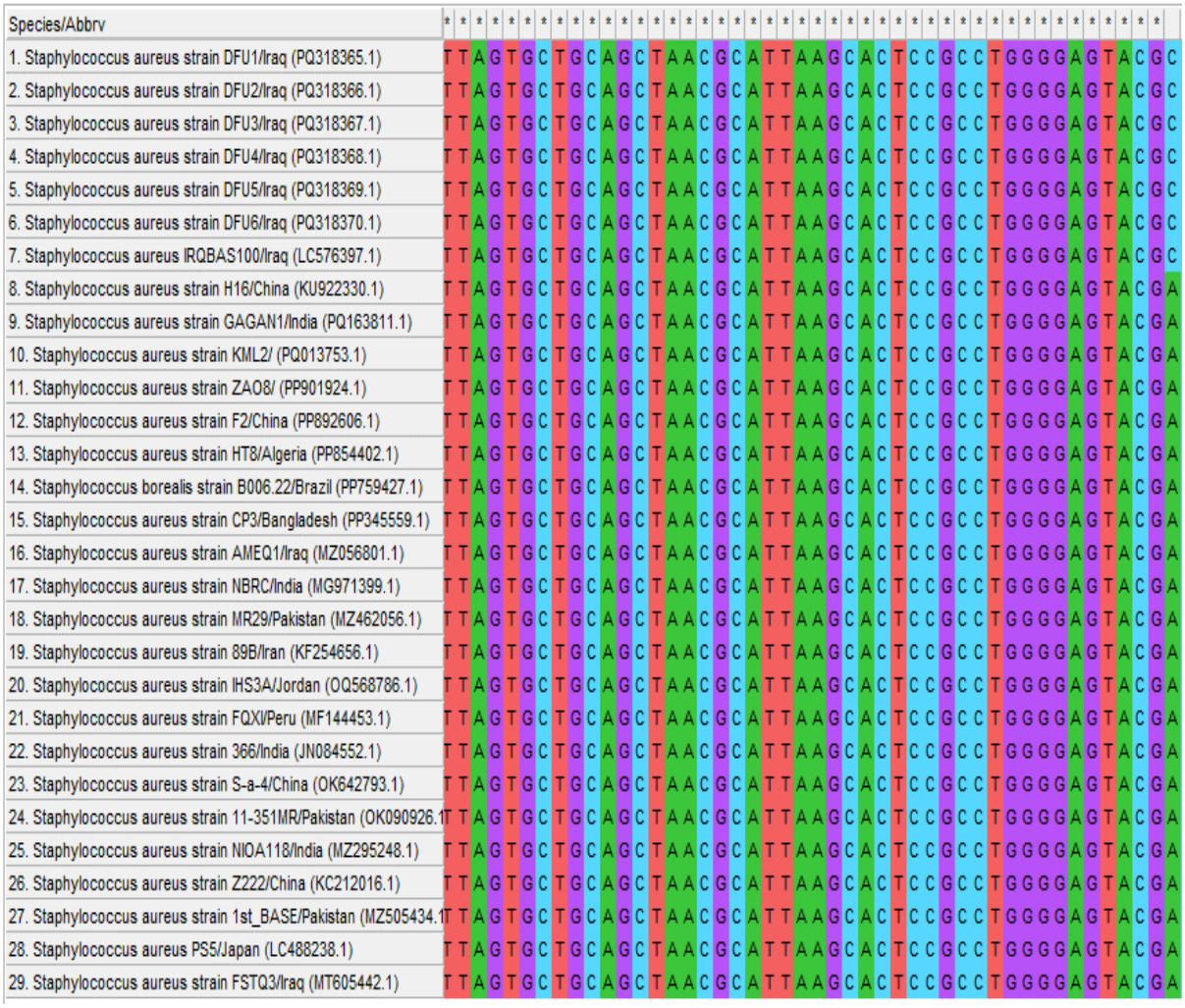


**Figure (3): Multiple sequence alignment of the local and NCBI-BLAST *S. aureus* isolates/strains using of MEGA-11 software**

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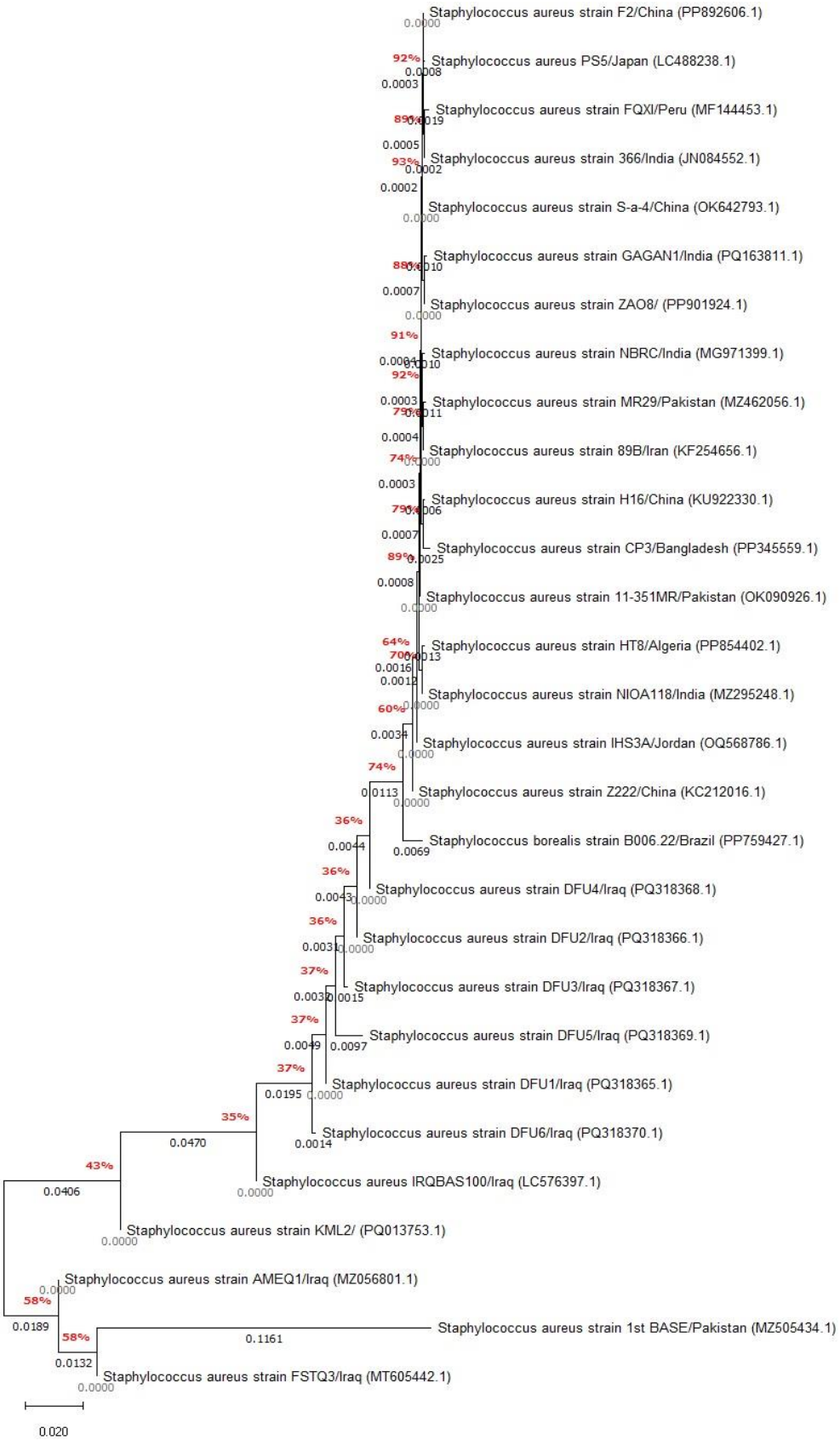


**Figure (3): Phylogenetic tree analysis of the local and NCBI-BLAST *S. aureus* isolates/strains using of MEGA-11 software**

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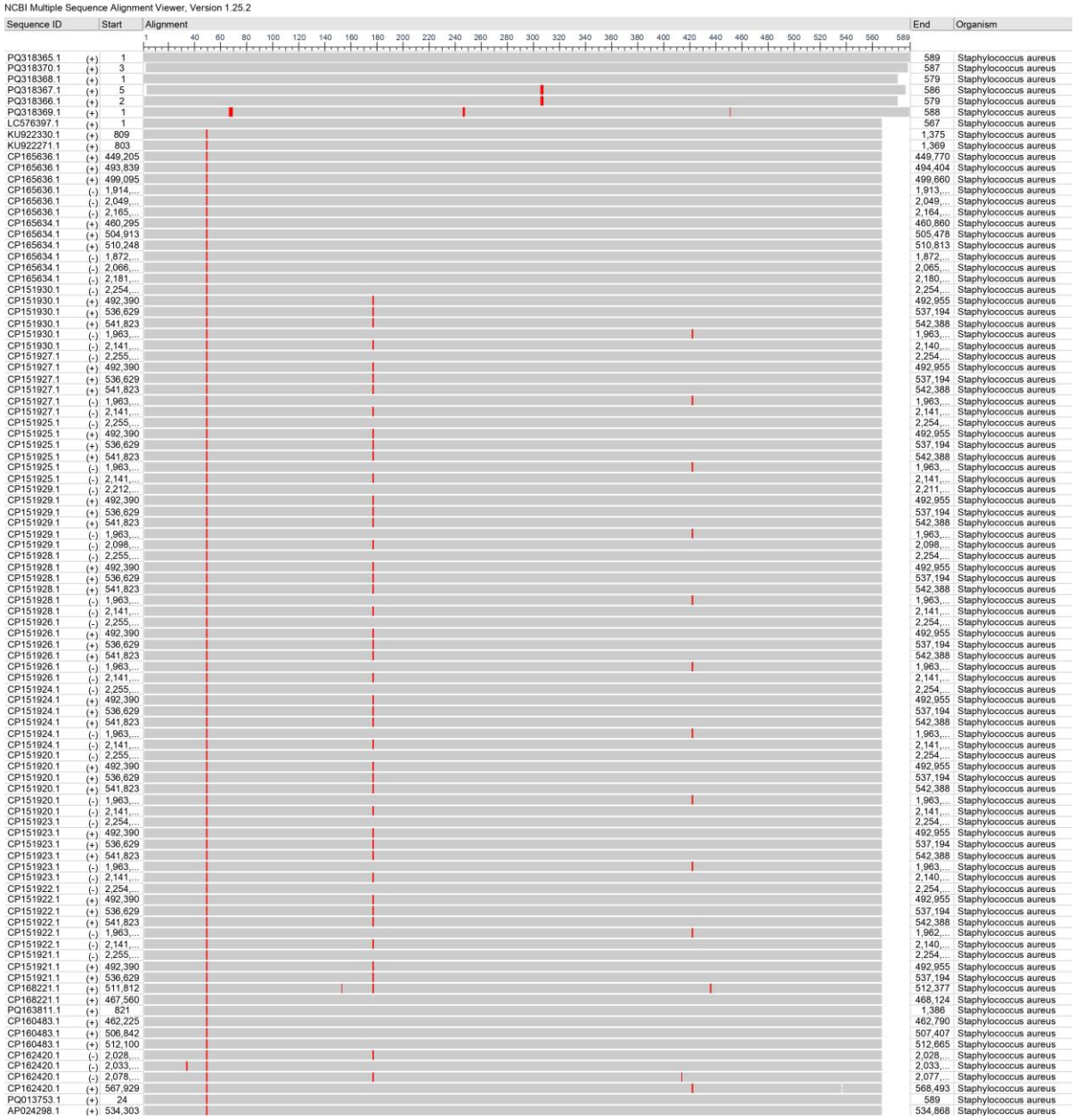


**Figure (5): Multiple sequence alignment of the local and NCBI-BLAST *S. aureus* isolates/strains using NCBI MSA Viewer**

**Table (2): Homology Sequence identity (%) for local and NCBI-BLAST *S.aureus* isolates**

| **Local isolate** | | **NCBI isolate** | | | |
|---|---|---|---|---|---|
| **Name** | **Access No.** | **Species** | **Country** | **Access No.** | **%** |
| DFU1 | PQ318365.1 | *S. aureus* | Iraq | LC576397.1 | 99.72 |
| DFU2 | PQ318366.1 | *S. aureus* | Iraq | LC576397.1 | 99.51 |
| DFU3 | PQ318367.1 | *S. aureus* | Iraq | LC576397.1 | 99.79 |
| DFU4 | PQ318368.1 | *S. aureus* | Iraq | LC576397.1 | 98.66 |

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| | | | | | |
|---|---|---|---|---|---|
| DFU5 | PQ318369.1 | *S. aureus* | Iraq | LC576397.1 | 98.65 |
| DFU6 | PQ318370.1 | *S. aureus* | Iraq | LC576397.1 | 99.68 |

**Discussion**

Worldwide, several risk factors have been identified to predispose individuals with diabetes to development of foot ulcers. These include poor glycemic control, longer duration of diabetes, older age, obesity, smoking, and a history of previous foot ulceration or amputation. Foot deformities, such as Charcot foot, and inadequate foot care can also increase the risk of ulceration (Ahmad, 2016; Ntuli et al., 2018; Lin et al., 2020).

Different studies demonstrated that *S. aureus* was the more predominant Gram-positive pathogen, responsible for infections of mild-to moderate severity in diabetic patients with foot ulcers (Esposito et al., 2008; Bengalorkar, 2011; Appapalam et al., 2021). Dunyach-Remy et al. (2016) described the role of S. aureus in DFUs and the implication of its toxins in the establishment of infection. In this study, PCR assay demonstrated the presence of *S. aureus* infection in ulcers of 20.55% DFU patients, which in agreement with that reported previously in India (24.5%) by Shankar et al. (2005) and recently in Iraq by Hussein and Saleh (2024) who identified *S. aureus* in 24.28% of the DFU patients attended to two hospitals in Alsamawa and Baghdad provinces during the 2022s. In comparison to other global studies, a study conducted by Tentolouris et al. (2006) in Greece has detected 18 out of 91-foot ulcers were infected by *S. aureus* in 19.78%, in which, methicillin-resistant *S. aureus* (MRSA) isolates were more common among patients with infected foot ulcers (61.1%). Eleftheriadou et al. (2010) showed that the prevalence rate of MRSA in DFUs was 15-30%, and there is an alarming trend for increase in many countries. In 2014, a study conducted in USA characterized 25 different *S. aureus* isolates in DFU patients, and most of these isolates were resembled genetically to those isolated from patients with atopic dermatitis (Vu et al., 2014). Shettigar et al. (2016) detected that 43% (86/200) of DFUs were infected with *S. aureus*, and virulence genes were found in 94.19% (81/86) isolates, with presence of more than one gene in 41 isolates. In a cross-sectional study among 83 adult DFU patients in Kenya, the findings showed that 94% of swab samples were positive to Gram-negative (65%) and Gram-positive (29%) with detection *S. aureus* in 16% of these isolates. In Iraq, the culture findings of Qadir et al. (2020) observed that the prevalence rate of Gram-positive bacteria was significantly greater than Gram-negative bacteria, and that *S. aureus* encompasses 54.7% of identified Gram-positive isolates. Another study recorded that 201 out of 863 (23.29%) DFU swabs were infected with S. aureus, in which, MRSA isolates were detected in 15.4% (Stańkowska et al., 2022). In a recent systemic review and meta-analysis encompassed 40 studies cross 20 countries, Zhou et al. (2024) found that the overall prevalence of MRSA in DFU was 17% with significant regional variation; 61% in South America, 20% in North America, 19% in Europe, 13% in Africa and 11% in others. However, the uncontrolled use of antibiotics in both hospitalized and non-hospitalized patients may contribute effectively to increasing the prevalence and risk of *S. aureus* among the DFU patients.

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Variable prevalence of *S. aureus* among different ages, sexes and medications has showed in the current study. The fact that more *S. aureus* isolates were seen in DFU patients older than 60 years may be due to either the high incidence of chronic diseases or attenuation of immunity in advanced aged. Also, various factors such as professional activities and lifestyle may cause the feet to tolerate more pressure, with increasing the incidence of consequences that are more commonly in older people. The main results of Cervantes-García et al. (2015) were included 34% positive *S. aureus* isolates and indicated that the grade-4 of DFU is more common in patients aged 54-65 and 66-88 years old and having diabetes for 11-15 years or longest period (16-30 years). Subsequently, the authors were found no significant variation in prevalence different microbes isolated from DFU lesions, but showed that majority of had a localized gangrene, and majority of women having deep ulcers with cellulitis and / or abscess in the skin. Mohammed et al. (2016) mentioned that older age, long diabetes duration, multiple anti-diabetics, hard physical activity, and bad foot care are the common risk factors for the development of DFUs. Lee et al. (2017) showed that the MRSA infection was not differed between DFU patients of both age groups, ≥65 (14.7%) and <65 (15.8%) years as well as between males (15%) and females (161%). In an Iraqi study, Qadir et al. (2020) identified that DFU was more common in individuals aged 50-59 years and to less extent in 60-69 and ≥70 years old when compared to <40 and 40-49 years old. Also, they recorded that DFU is more related with oral drugs than insulin and mixed (oral drugs and insulin) medications. A cross-sectional study conducted by Anafo et al. (2021) found that the prevalence rate of S. aureus among DFU patients was 19%, and infection is more common in advanced ages, 30-60 years (42%) and >60 years (57%), as well as in females (54%) more than males (46%). Among totally 1771 patients, Vanherwegen et al. (2023) reported that the vast majority of DFU infection was seen in males (72%), and lesions in males being deeper, more frequently, displaying probe to bone and more frequently deeply infected, while women had a 265 higher chance of healing without major amputation as a first event.

*Staphylococcus aureus* is characterized by both vertical and horizontal evolution, and the vertical historical signal is often strong enough to make phylogenetic reconstruction meaningful and useful (Planet et al., 2017). In the current study, phylogenetic analysis revealed that the local *S. aureus* strains were highly identical to the NCBI-BLAST *S. aureus* strains isolated from eye infection of human at Basra province (Iraq) and submitted in the NCBI-GenBank in 2020. These findings support the fact that *S. aureus* can cause different skin and skin structure infections including DFUs, osteoarticular and ocular infections, pneumonia and endocarditis (Isah, 2021; Van Dorpe, 2021). However, genotyping methods add precision in the identification of S. aureus, and partial 16S rRNA sequencing can be served largely in identification of different pathogenic isolates / strains (Kosecka-Strojek et al., 2019; Wilson et al., 2019).

## Conclusion

This study confirms, for the first time in Iraq, the phylogenetic linkage of *S. aureus* isolated from DFU patients to the NCBI-GenBank *S. aureus* isolates. This study shows, also, significant differences in prevalence rates and risks of *S. aureus* among various age and sex groups as well as among DFU patients received different medications. Therefore, establishing

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the underlying causes of diabetes and developing effective interventions to prevent and manage its associated complications are crucial steps towards reducing the burden of this widespread disease. Additionally, the high incidence and recurrence rates of DFU remain underscore and need for a multifaceted approach to prevention and management.

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**Conflicts of interests**

Author declares no conflict of interest.

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