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# Molecular Detection of Virulence Factors Genes for *Staphylococcus aureus* in Diabetic Foot Ulcers in Iraq

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## Abstract

One of the main complications in patients with Diabetes Mellitus is diabetic foot ulcers (DFUs), a significant and common disease. The wound of a diabetic foot reduces vascular supply and decrease host immune response, facilitating bacterial infection. The pathogen that is most commonly isolated in DFUs is *Staphylococcus aureus*. The purpose of this work was to identify *S. aureus* by conventional, biochemical, and molecular detection of the virulence factor genes in DFUs. This case cohort study was directed at Al-Hussein Teaching Hospital (AIsamawa, Iraq) and Medical City/Baghdad Hospital (Baghdad, Iraq) from January to July 2022, and it involved 140 patients with diabetic foot ulcers. Extraction of bacterial DNA from the swab samples was done by ordinary microbiology techniques and was used to achieve polymerase chain reaction (PCR) using specific gene primers (16S ribosomal RNA, *mecA*, *pvl*, and *α-hly*). *S. aureus* was identified in 34 (24.28%) of the samples. 73 (52.14%) positive cultures of bacterial growth versus 67 (47.85%) show negative results for culturing. The concentration of DNA extracted from 140 swab samples ranged from 10 to 153 ng/µl. 16S rRNA showed that all 34 isolates gave positive results. PCR results were included. 31 (29.8%) of the *Staphylococcus aureus* were hiding the *mecA* gene. *The α-hly* gene was found in 30 (28.8%) of the *S. aureus* positive samples. *The pvl* gene was only present in 10 (9.6%) of *S. aureus* isolates found in DFU's samples. In DFUs, *S. aureus* predominated among the microorganisms. All *S. aureus* isolates included in this investigation had a high prevalence of the 16S rRNA gene. It serves as a genetic marker that can be utilized to confirm isolates of *S. aureus*. *MecA*, *pvl*, and *α-hly* genes can be found via PCR analysis.

**Keywords:** Diabetic foot ulcer, *Staphylococcus aureus*, Polymerase chain reaction, 16S rRNA, *mecA*, *pvl* and *α-hly*.

## 1. Introduction

Hyperglycemia is a metabolic disease defined by hyperglycemia caused by insulin action, secretion, or both. In approximately 15% of diabetics, a diabetic foot ulcer appears as an open sore. They're usually seen around the bottom of the foot. Diabetic foot ulcers are a consequence of poorly managed diabetes. Muscle weakness, foot deformities, peripheral neuropathy, and neuropathic ruptures are all possible causes [1–3].

*Staphylococcus aureus* is a Gram positive, spherical, facultatively anaerobic, and devious pathogen that inhabits the skin and mucosal surface of the human host [4,5]. *Staphylococcus aureus* is the most common bacterial isolate described in Diabetic foot infection (DFI) from western countries, causing delayed wound healing. Virulence factors such as proteases, lipases, nucleases, and hemolysins, which make host tissues promising for bacterial growth and tissue attack, enhance wound adherence, persistence, and infection. Because the extent of *S. aureus* in soft tissues and bones can lead to amputation of the lower ends, early identification and good wound treatment are crucial. The virulence potential of each bacterial species varies in the wound environment, so it's critical to assess the essential virulence elements of isolated types to describe and discriminate between pathogens that cause infection [6, 7].The staphylococcus cassette chromosome methicillin-sensitive *S. aureus* (SCCmec) *MecA* gene is in the variety of a specific chromosome, and methicillin resistance is conferred by the *mecA* gene, which codes for penicillin-binding protein 2a (PBP2a). This initiates the reduction of binding and attraction for *B*-lactam antibiotics, including penicillinase-resistant penicillin. PBP is a family of enzymes found in the cell membrane of *S. aureus* that catalyzes the trans-peptization required for the synthesis of peptidoglycan chains [8].The staphylococcus cassette chromosome methicillin-sensitive *S. aureus* (SCCmec) *MecA* gene is in the variety of a specific chromosome, and methicillin resistance is conferred by the *mecA* gene, which codes for penicillin-binding protein 2a (PBP2a). This initiates the reduction of binding and attraction for *B*-lactam antibiotics, including penicillinase-resistant penicillin. PBP is a family of enzymes found in the cell membrane of *S. aureus* that catalyzes the trans-peptidation required for the synthesis of peptidoglycan chains [8].*The alpha* (*α*) gene is found on the bacterial chromosome, and it codes for one of the most well-known pore-forming toxins. Exotoxin is a secreted protein with hemolytic, cytotoxic, dermonecrotic, and deadly characteristics. Practically all clinical isolates of *S. aureus* produce this 33 kDa pore-forming toxin as a monomer. Despite the fact that α-toxin is potent against an extensive variety of mammalian cells, it has species and cell type specificity [9, 10]. Panton-Valentine leukocidin (*pvl*) is a very toxic cytotoxin. This toxin belongs to a group of proteins that produce membrane holes. It is made up of two discrete protein constituents, LukS-PV and LukF-PV [11]. Dermonecrosis, chronic skin and soft tissue infection (SSTI), chronic mucocutaneous infections, and necrotizing pneumonia are all linked to the active toxin, which causes neutrophil lysis by developing a hole in their membrane [12, 13].

## 2. Materials and Methods

### 2.1.Isolation and identification of *S. aureus*

The samples involved 140 swabs from diabetic foot ulcers collected over seven months between January and July 2022, at Al-Hussein Teaching Hospital (Alsamawa, Iraq) and Medical City, Baghdad Hospital in Baghdad, Iraq. Swab samples were collected by transport media swabs, which were taken from an infected area of the diabetic foot. All these samples were cultivated on plates of Mannitol Salt Agar (MSA) and Blood Agar (BA). Then incubated at 37°C for 24 hr.; after growth was observed, a Gram stain was carried out, and biochemical experiments were performed needed for identification of *S. aureus* from other species.

### 2.2. Detection of 16SrRNA, *α-hly*, *mecA* and *pvl* genes

#### 2.2.1. Extraction of Bacterial DNA

Using the Presto$^{TM}$ Mini gDNA Bacteria (Geneaid/Taiwan) Kit, *S. aureus* entire genomic DNA was extracted and purified following the instructions provided by the kit's company. By using the Nanodrop (Thermo Scientific NanoDrop Lite UV Visible Spectrophotometer, USA), which

measures DNA concentration (ng/L) and examines DNA quality at absorbance (260/280 nm), the extracted total DNA was examined.

### 2.2.2. Polymerase chain reaction (PCR) technique protocol

A polymerase chain reaction (PCR) system was carried out to augment fragments of 16SrRNA (474bp), *α-hly* (209bp), the mecA gene (147 bp), and *the pvl* gene (433bp) genes, as shown in Table 1. The manufacturer (Macrogen/Korea) provided the primers, as shown in **Table 1,** as lyophilized powder in Eppendorf tubes (1.5ml). Before opening, the tubes were centrifuged at 8000 rpm for 5 seconds to settle the lyophilized primers. Next, 1X TE buffer (pH 8.0) was added to all tubes at the manufacturer's recommended volume in order to prepare a stock solution concentration of 100 ml/µl. The working solution of the primers was made by adding 10µl of primer stock solution (which was saved at -20°C) to 90 µl of nuclease free water solution to get a working primer solution (10ml/µl). The PCR mixture was achieved in a total size of 25 µL and controlled the DNA template (25 µL), primers (4 µL) specific for the 16SrRNA, *α-hly*, *mecA*, and *pvl* genes, nuclease-free water (3.5µL), and 12.5µL of premix (2X) with the thermocycling conditions set at one cycle of 95°C for 5 min, followed by 35 cycles of 95°C/30 s, 58°C/30 s, and 72°C/1 min, and one cycle of 72°C/5 min, then 4°C of stop step. PCR products were visualized using 5% agarose and TBE buffer (0.5x) at 100 volts and 80 amps at 70 volts for 1 hour (Promega, USA). All primers for PCR for the 16S ribosomal RNA gene *S. aureus* were chosen in this study using the National Center Bank Institute NCBI Genbank database. *MecA*, *PVL*, and *α-hly* genes were identified in *S. aureus* based on sequences published by [14–16], respectively. These primers were provided by Scientific Researcher. Co. Ltd. in Iraq. The sequences of all primers were checked out depending on the National Center Bank Institute (NCBI) Database.

**Table 1.** Sequence of the primers was used with the name and size of the product.

| Genes | Primer | Nucleotide sequences 5' ⟶ 3' | Product size(bp) |
|---|---|---|---|
| *mecA* | mecA-F | GTGAAGATATACCAAGTGATT | 147 |
| | mecA-R | ATGCGCTATAGATTGAAAGGAT | |
| *Pvl* | Luk-PV-1 | ATCATTAGGTAAAATGTCTGGACATGA TCCA | 433 |
| | Luk-PV-2 | GCATCAAGTGTATTGGATAGCAAAAGC | |
| *α-hly* | Fw | CTGATTACTATCCAAGAAATTCGATTG | 209 |
| | Rv | CTTTCCAGCCTACTTTTTTATCAGT | |
| 16S rRNA | F | TCAACCGTGGAGGGTCATTG | 474 |
| | R | CCCAACATCTCACGACACGA | |

## 3. Results

### 3.1. Isolation and identification of *S. aureus*

Clinical specimens were collected from 140 DFU patients at Alsamawa and Baghdad Teaching Hospitals and streaked on Mannitol salt agar. 34 isolates looked like round yellow colonies and were positive for the catalase test; consequently, they are predominantly recognized as *S. aureus*.

### 3.2. DNA extraction and preparation

Following DNA extraction using the Presto™ Mini gDNA Bacteria Kit, the DNA concentration was 10–153 ng/µl. Gel electrophoresis was prepared to check the purity of the extracted DNA. Polymerase chain reaction detection of 16SrRNA, *mecA*, *α-hly*, and *pvl* genes. All 34 strands of DNA of *S. aureus* were submitted for molecular identification using the 16S rRNA gene as specified in **Table 1** with the PCR technique.

**Table 2:** Frequency of virulence genes of *S. aureus* isolates in DFUs.

| | ***mecA*** | | ***α-hly*** | | ***pvl*** | |
|---|---|---|---|---|---|---|
| | N | Percent | N | Percent | N | Percent |
| Positive | 31 | 29.8 | 30 | 28.8 | 10 | 9.6 |
| Negative | 3 | 2.9 | 4 | 3.8 | 24 | 23.1 |
| P value | <0.0001* | | <0.0001* | | <0.016* | |

The 16S rRNA gene was employ as a genetic marker to confirm identification of *S. aureus* isolates and it has become more common in the clinical environment [17]. Our results of amplifying 16S rRNA was given positive results in all isolates 34(34%) of *S. aureus*, **Table 2** and single band in 474bp amplicon size, **Figure 1A**.

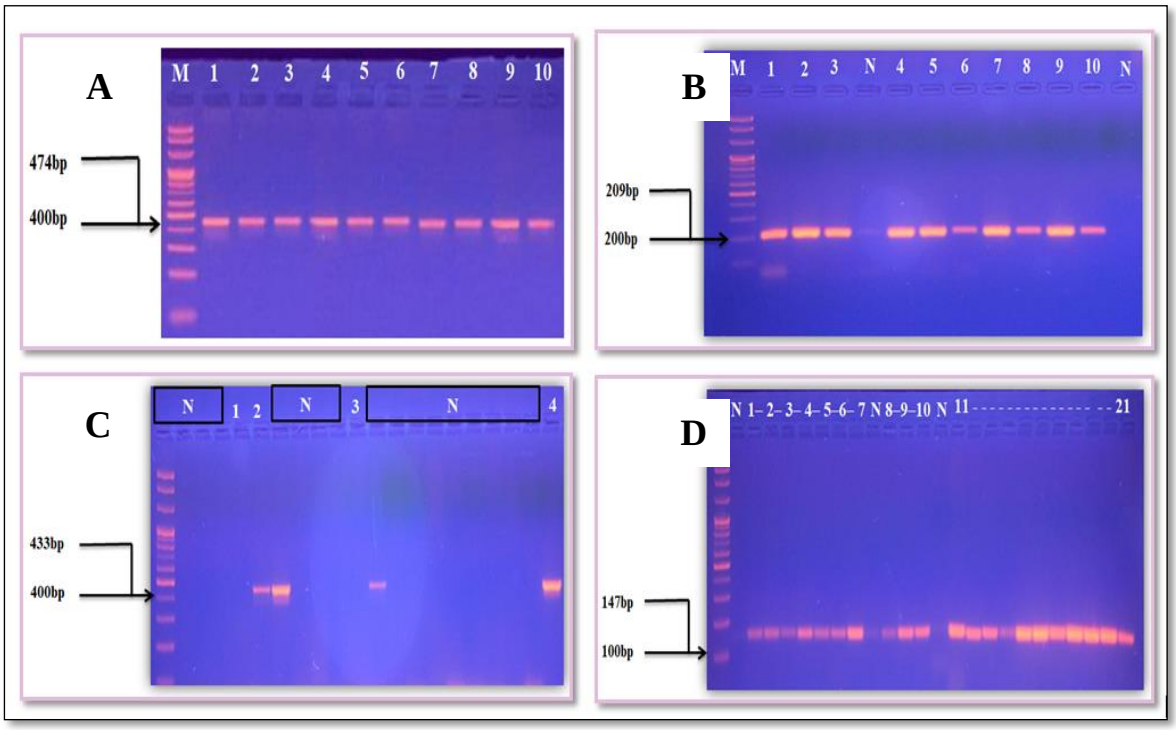


**Figure 1:** Gel electrophoresis of the PCR product. (A) 16SrRNA gene in *S. aureus* with an amplicon size of 474 bp Line M: DNA marker (100-1500bp); Lines 1–10: Positive isolates of *S. aureus.* (B) *α-hly* gene in *S. aureus* with an amplicon size of 209 bp. Line M: DNA marker (100-1500bp); Lines 1–10: Positive isolates of *S. aureus*. (C) *pvl* gene in *S. aureus* with an amplicon size of 433 bp. Line M: DNA marker (100-1500bp); Lines (1, 2, 3, and 4): positive isolates of *S. aureus*. (D) *mecA* gene in *S. aureus* with an amplicon size of 147 bp. Line M: DNA marker (100-1500bp); Lines 1–11: Positive isolates of *S. aureus*. Lines N in A,B,C and D: Negative isolates of *S. aureus* migrated in 5% agarose, TBE buffer (0.5x), and current 200A at 70 volts for 90 minutes.

The results of *α-hly* revealed that *the α-hly* gene was identified in 30 isolates (28.8%) **Table 2**, which revealed bands of 209 bp as shown in **Figure 1B.** *A-toxin* is a major virulence component of *S. aureus* that has been significantly related to skin and soft tissue infections in humans. Most *S. aureus* strains release this pore-forming toxin, which is predominantly composed of beta sheets and targets red blood cells [6]. Current results revealed that the pvl gene (433 bp) in **Figure 1C** was identified only in 10 (9.6%) isolates out of 34 isolates in Table 2.A single band of the *mecA gene was* observed at the given molecular weight (147 bp), as shown in **Figure 1D.** The results revealed that *the mecA* gene was identified in 31 (29.8%) out of 34 isolates **Table 2.**

## 4. Discussion

Our results of amplifying 16S rRNA all isolates 34(34%) of *S. aureus* agreeing with [18] that found the outcome of amplifying 16S rRNA was revealed the all 18 isolates presented positive results. However, [15] discovered that 60 of 67 *S. aureus* isolates possessed 16SrRNA. Despite the fact that seven isolates were recognized as *S. aureus* using conventional techniques, they lost this gene. Current results found that almost all isolated strains contain *the α-hly* gene 30 (28.8%). This is compatible with the French's study, which reported that nearly all of the strains had the α-toxin-encoding gene hla freely of the grade [19]. Alpha-toxin production was also evaluated among the 25 DFU isolates [20]. Additionally, [21] the *α*-toxin gene was found in 85 percent of the *S. aureus*-positive specimens. The outcomes of the existing study agreed with those of another study done by [15] in Baghdad, which showed the detection of the exotoxin *pvl* gene in only 6 (10%) isolates out of 60 isolates offered a positive consequence. Also, [22] revealed that 19 (79%) out of 24 isolates had a positive outcome for *the pvl* toxin gene. While a study showed that amplifying *the pvl* gene was indicated in all 18 isolates and gave a positive outcome [18], *Pvl* is an extracellular protein that serves as a dermonecrotic and Leukocidin agent [23, 24, 25, 26]. The *mecA* gene produces penicillin-binding protein (PBP2a), which is responsible for methicillin resistance in MRSA and is capable of decreasing affinity for β-lactam antibiotics [27, 28, 29]. While the *mecA* gene results by [15] showed that the mecA gene was located in 48 isolates (80%) of all *S. aureus* isolates, [30] reported that *mecA* frequency was 96% among *S. aureus* isolates, which was consistent with our result.

## 5. Conclusion

In DFUs, *S. aureus* predominated among the microorganisms. All 34 *S. aureus* isolates involved in this investigation had a high prevalence of the 16S rRNA gene. *MecA*, *pvl*, and *α-hly* genes can be found via PCR analysis. *The MecA* gene was higher than other genes.

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## Conflict of Interest

The authors declare that they have no conflicts of interest."

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## Ethical Clearance

This study involving human participants was reviewed, and the patients and participants provided their written consent to contribute to this paper.

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