Cytotoxin Production and Slim Layer Formation by MRSA *Staphylococcus auras* Isolated from Diabetic Patients

Abstract

Methicillin-resistant A frequent infection linked to diabetic foot ulcers is Staphylococcus aureus (MRSA).There are no investigations on the connection between MRSA and the neoplasia of perivascular tissues and the delayed healing of diabetic foot ulcers. The purpose of this study is to genetically isolate MRSA from DFUs patients and define its function in the development of chronic ulcer lesions, the course of the disease, and antibiotic resistance. Only (31) isolates were found in the (80) samples taken from diabetic foot ulcer patients admitted to the Diabetes Endocrinology Center: 12 Staphylococcus aureus (38.7%) and 19 other Staphylococcus spp. (61.3%). Staphylococcus aureus stain pak2, MR30, CFSAN007896, and ST4 were the results of amplification of the universal 16S rRNA gene, which confirmed all 12 (100%) of the samples. The mecA gene revealed 100% positive results for the icaA gene, which is involved in biofilm formation, and 9 (75%) positive results for the pvl genes, which are involved in the manufacture of cytotoxins, proving that all 12 Staphylococcus aureus isolates were methicillin resistant (MRSA). The findings demonstrated that the high prevalence of PVL among MRSA strains isolated from DFUs that form biofilms causes patients to have significant inflammation, illness progression, and challenging wound healing, which may result in lower limb amputations.

Keywords: diabetic foot ulcers (DFUs); Methicillin resistance *Staphylococcus aureus (MRSA);* Cytotoxin ; Biofilm ; universal *16SrDNA* gene.

Introduction

Diabetic foot ulcers (DFUs) are characterized by a number of pathological sequelae, such as neuropathy and peripheral vascular disease. **(1. 2).**

Staphylococcus epidermidis, *Staphylococcus aureus*, and *Propionibacterium acnes* are a few examples of pathogenic bacteria that can cause infection. They can also be present in the skin's physiological microflora. Almost any germ that comes into touch with the surface of an ulcer is likely to colonize it. Medical personnel, supplies, and drugs used for therapy frequently unwittingly spread harmful microflora. A variety of bacteria strains are often present in the mixed flora of ulcers **(3, 4).** In addition to producing slim layer, *S. aureus* involve in the infection of soft tissues and bones. In fact, slim layer (glycocalyx or biofilm) forming bacteria that infected foot ulceration is the cause of 80% of lower-limb amputations. Toxins, which can cause tissue necrosis, progression and spread of infection in DFIs patients, can also be released by *S. aureus* in addition to basic adhesion mechanisms (**5, 6, 7**).Methicillin-resistant *S. aureus*, which accounts for the majority of *S. aureus* infections, is present in 10–40% of diabetic wounds and produces Panton-valentine leukocidin (PVL), a cytotoxin that is crucial to MRSA virulence (**8,9).**

The goal of this study is to identify *MRSA* (methicillin-resistant *Staphylococcus aureus*) genetically from DFUs patients and discuss its function in: Biofilm formation and identifying the genes that are the primary contributors to delayed healing and lower limb amputations, Producing the cytotoxin (pvl), the primary cause of tissue necrosis, and assessing the frequency of its genes in *MRSA* isolated from infected DFUs patients.

Materials and Methods

1 Morphological and Biochemical Characteristics

A total of 80 swabs from the necrotic lesions of patients with diabetic foot ulcers (DFUs), whose cause was complex diabetic foot syndrome (DFS), were obtained and evaluated under the supervision of a doctor. In Al-Basrah, southern Iraq, the main hospitals (Al-Faiha General Teaching Hospital and Al-Mawanaa General Teaching Hospital) treated and admitted patients to the Diabetes Endocrinology Center for the 2019–2020 academic year. All of the patients had type 2 diabetes (T2DM), which had an average duration of 14.5 ± 2.5 years. The patients were between the ages of 40 and 55, plus or minus five years. These swabs were then cultured, first in sterile tubes with 5 ml of Brain Heart Infusion Broth-BHIB (HIMEDIA/India) and then moved to the laboratory, incubate 24 h. at 37 °C. The Staphylococcus Chromogeneic Agar Media (CONDA Pronadisa/spain) was streaked after the broth media showed positive growth.After being cultivated on nutrient agar (HIMEDIA/India) for 24 hours at 37 °C, colonies developed (**10).** Gram staining was done to identify the colonies.

Congo Red Agar (CRA) method: -

This agar was made by mixing blood agar base with 0.8 gm congo red, 50 gm sucrose, and 1 litter distilled water. The pH was then adjusted to 8 and the agar was autoclaved at 121 C for 15 minutes. According to Freeman **(11).**

2-Bacteria 16s rDNA Genotyping

Extraction of DNA

The DNA extraction was done by genomic DNA micro kit (Geneaid, Taiwan).

I6S rDNA amplification and sequencing

In order to identify the bacterial strains, the extracted DNA was processed through PCR to amplify the universal bacterial 16SrDNA gene, which is represented by the sequences B 27 F (5'-AGAGTTTG ATCCTGGC-3') and U 1492R (5'-GGTTACCT TGTTACGACTT-3').92 °C for 2 minutes, then 30 cycles of 94° C for 30 seconds, 51° C for 45 seconds, 72°C for 1.5 minutes, and 72 °C for 5 minutes **(12)**.

The positive samples of PCR products for universal *16s rDNA* gene were sent to Bioneer Company (Korea) for sequencing for more identification.

16S rDNA identification

The 16s rDNA sequences of the bacterium isolates were compared to the Genbank 16s rDNA reference database (http://blast.ncbi.nlm.nih.gov/).

Detection of the *mecA* , *icaA,* and *pvl* genes

The *mecA* gene is used to identify methicillin-resistant *Staphylococcus aureus* species using the primers *MecA1* and *MecA2* according to (**13**), and Luk-PV-2 depending on (Zhang *et al*. 2004).**While** slime layer is encoded by the *icaA* gene, which has the primers *icaA1* and icaA2 according to **(14)** and the *pvl* genes using the primers *Luk-PV-1* and *Luk-PV-2* to detect the PVL S/F bicomponent proteins depending **(15).**

MecA genes were subjected to a set of thermocycling conditions that included three minutes at 95°C. followed by 30 cycles of one minute each at 94 °C., 53 °C., one minute at 72°C, and six minutes at 72°C. *IcaA* genes were subjected to a set of conditions that included five minutes at 95 °C. followed by fifty cycles of one minute each at 94 °C. five minutes at 55.5 °C., one minute at 72 °C., and one minute at 95°C.

Results

1-Morphological and Biochemical Characteristics

Staphylococcus aureus was isolated and found to be dependent on its color (pink to mauve) on Staphylococcus Chromogenic agar media. Out of (80) swab samples, only (31) isolates, including 12 (38.7%) isolates were identified as *Staphylococcus aureus*, while the other 19 (61.3%) isolates were identified as *Staphylococcus* spp.

I6s rDNA amplification and sequencing

The PCR products for the extracted DNA from 12 isolates resulted in (100%) positive results, and when they were seen under a UV light source and compared to a DNA ladder, they showed a single band at a location of 1500 bp on an agarose gel. Using the Basic Local Alignment Search Tool (BLAST), 7 (87.5%) strains of *S. aureus* from 12 isolates were found. These strains were then compared to a reference strain from Gene Bank that was the exact same strain. These include the *S. aureus* strains pak2 (n=5), MR30 (n=3), CFSAN007896 (n=3), and ST4 (n=1).

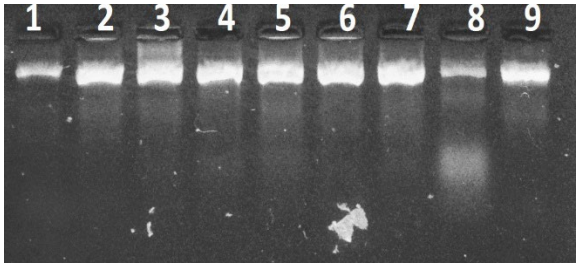

Fig.١. Electrophoresis of agarose gel (0.8%) for I bands **.**

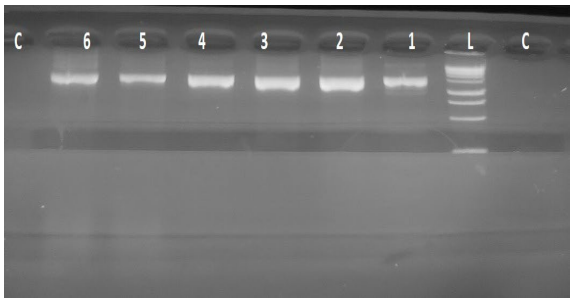

Fig.٢. Electrophoresis of agarose gel (1%) for Universal*16SrDNA* PCR products at position 1500bp from (1-6) , L: (Ladder:250-10000bp).

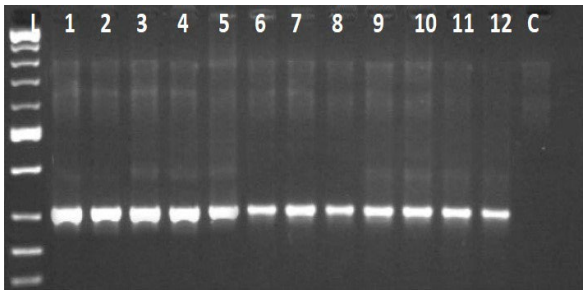

Fig.٣. Electrophoresis of agarose gel (1%) from (1-12) specific *mecA* gene products at position 310 bp, L: (Ladder:100-1000bp).

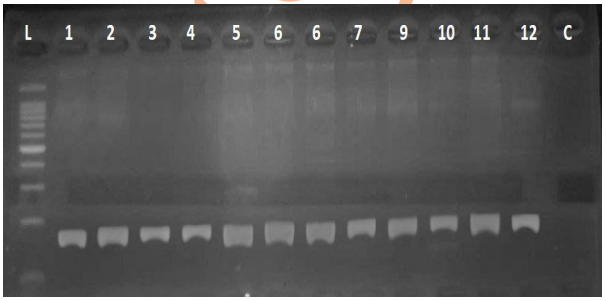

Fig.٤. Electrophoresis of agarose gel (1%) from (1-12) specific icaA gene products at position 188 bp, L: (Ladder:100-1000bp).

Bacterial strain	Identical wi reference Strain (%)	Nucleotide sequence
Staphylococcus aureus strain pal 706 bp n=5	100%	CTGTGCAATCTTGACGGTACCTAATCAGAAAGCCACGGCTA ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA GGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAC TGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGA CTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATC TAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGC AGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAC TGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCG AACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGT GCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCG GGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGC GAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACC ATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCC CGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCT TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC AAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGTTGAC CGGTGACAAAC

Table 1. Alignments of *PCR* product for*16SrDNA* gene

Staphylococcus aure strain *MR30* 660bp n=3	100%	GGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG GTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAA CGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCT ACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGA AAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGG GAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCT TGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAG CACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCC TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG CGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGC TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCA TCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCCCTTC GGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCG AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTA TGCCATCATTAAGTT
Staphylococcus aure strain CFSAN0078 706bp n=3	100%	AGTCCAGTAACTGTGCACATCTTGACGGTACCTAATCAGAAAGC CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA GTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTT AGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGG ACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTC GCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGC ACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGA AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG CTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGC TAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAA
Staphylococcus aure strain ST4 676bp n=1	100%	GAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAAT GAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACA TCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTG GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC GCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGG ACTCTAAGTTGACTGCCGGTGCTACCAAAAAAAAAAAAAAAAA AAAAATATGTATAAA
		TCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCA AGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTG CGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGC

CGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTG
GAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGA
TATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAAC
ACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACC
GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTT
CGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG
TACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
ACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTC
TTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTC
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTA
CTTAGTTGCCATCATTAAGTTGGGCACTCT

Results for the *mecA*, *icaA*, and *pvl* genes

Amplification of the *mecA* and *icaA* genes produced bands at positions 310 bp and 188 bp, respectively, as shown in Figures ٣, ٤, and ٥. Nine (75%) of the 12 isolates encode for the *pvl S/F* gene (Bicomponent proteins), which produced bands at position 433 bp in Figure ٤.

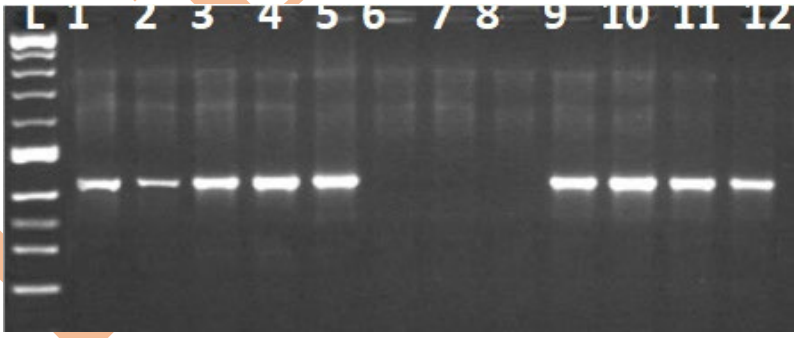

Fig .٥ . Electrophoresis of agarose gel (1%) from (1-9) specific *pvl S/F* gene products at position 433bp, L: (Ladder:100-1000bp).

4.Discussion

The present work examined CHROMagar as a direct isolation medium for specimen isolation, enabling simple differentiation of bacterial colonies based on color and shape characteristics on CHROMagar. As a result, when mixed pathogens were grown on the medium, the medium's capacity to detect them was enhanced (**16, 17)**. With the help of universal 16S rDNA primers (F27 and R1492), the collected DNA was

amplified. The primers avoid the loss of any potential or new bacterial strains by amplifying the *16S* rDNA (16S ribosomal RNA) gene for all bacterial strains (**18, 17,19).**

Out of 80 patient swab samples, this study found that the prevalence of *Staphylococcus aureus* was 12 (38.7%), which is consistent with research done in Babylon, Iraq (**20)** and Bandar Abbas, southern Iran (**21)**. Additionally, this study's prevalence of MRSA was 100% of *mecA* in locations where methicillin resistant s. aureus (MRSA) was found using molecular techniques. This percentage was found in the study, which is greater than the figures found in the United States (29.8%) and Portugal (24.5%) according to (**22,23**).

The widespread and unchecked use of antibiotics in Iraq, which has led to significant levels of multi-drug resistance, is one of the country's biggest problems. Additionally, a lot of patients self-medicate with antibiotics, especially broad-spectrum antibiotics, without consulting a doctor. Therefore, we believe the high prevalence of MRSA in *S. aureus* isolates is the misuse of antibiotics, improper sterilization techniques, or the use of the incorrect sterilizers to treat ulcerated wounds. Any diabetic foot infection treated with MRSA will almost surely lead to an increase in resistance as well as a rise in medical expenses. All 12 MRSA isolates formed biofilm (100%) after samples underwent *icaA* and *pvl S/F* gene amplification, and 9 (75%) of them produced the cytotoxin (PVL), both of which are crucial for MRSA pathogenicity. The production and secretion of glycocalyx is necessary for *S. aureus* to be harmful, the neutrophil response is the first line of defense against *S. aureus* infection for strains derived from DFUs. Polysaccharide synthesis starts soon after attachment and starts to cover the bacteria. This is a crucial step in the formation of a biofilm that delays wound healing and increases biofilm thickness, especially in diabetic patients. Avoiding immune response by producing capsules or slime layers that conceal it and by inhibiting the death of phagocytes after ingestion (4, 24, 25).

In addition to biofilm formation, *S. aureus* contains a wide range of cytotoxins which have significant impact on the progression and spread of the bacteria in DFUs and has the potential to result in tissue necrosis (**26**) In this investigation, Most isolates from DFUs patients have *icaA* and *pvl* genes. This outcome is in line with research done in France and Iraq**.(27,7)**

The association between the MRSA strain's *IcaA* and *pvl* genes and the severe infection of diabetic foot ulcers, however, has not been studied. All MRSA isolated, (100%) developed the slime layer that retarded healing. Additionally, (75%) of 9 MRSA isolated produced the cytotoxin *pvl* that causes tissue necrosis. We then go on to describe how this virulence might cause foot amputation as a result of disease progression and sluggish wound healing.

5.Conclusion

Because MRSA strains positive for the *ICaA* and *PVL* genes are resistant to the majority of commonly used antibiotics, they are a significant contributor to severe infections, disease progression, chronic ulcer lesions, and difficult wound healing treatments, sometimes resulting in lower limb amputations. Additionally, our comprehension of how *IcaA* and *PVL* contribute to the inflammatory response to dangerous MRSA infections in diabetic foot ulcers has improved.

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