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Cytotoxin Production and Slim Layer Formation by Methicillin-Resistant *Staphylococcus auras* Isolated from Diabetic Patients

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ABSTRACT

Aims Methicillin-resistant *Staphylococcus aureus* frequently causes infection of diabetic foot ulcers. However, no investigations have investigated the connection between methicillin-resistant A, perivascular tissue neoplasia, and delayed healing of diabetic foot ulcers. The purpose of this study was to genetically isolate methicillin-resistant from diabetic foot ulcer patients and define its function in the development of chronic ulcer lesions, the course of the disease, and antibiotic resistance.

Materials & Methods This experimental study was conducted on patients referred to the Diabetes Endocrinology Center in Al-Basrah, Southern Iraq, from 2019 to 2020. Only 31 isolates were found in the 80 samples of diabetic foot ulcer patients; 12 *Staphylococcus aureus* (38.7%) and 19 other *Staphylococcus spp.* (61.3%). All 12 samples of *Staphylococcus aureus* strains were confirmed by amplifying the universal 16SrRNA gene for pak2, MR30, CFSAN007896, and ST4.

Findings The mecA gene was 100% positive for the icaA gene (involved in biofilm formation) and 75% positive for the pol gene (involved in the manufacture of cytotoxins), revealing that all 12 *Staphylococcus aureus* isolates were methicillin-resistant.

Conclusion Panton-valentine leukocidin has a high prevalence among MRSA strains isolated from diabetic foot ulcer patients that form biofilms, causing patients to have significant inflammation, illness progression, and challenging wound healing, which may result in lower limb amputations.

Keywords Diabetic Foot; Methicillin Resistance *Staphylococcus aureus*; Cytotoxin; Biofilm; Universal 16srdna Gene

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Introduction

Foot infections are a major cause of morbidity in diabetes patients and the most common cause of diabetes-related hospitalization and lower limb amputation [1]. The International Diabetes Foundation reports a significant increase in the number of individuals afflicted with diabetic foot ulcers (DFUs), estimated to be between 40 to 60 million people. This figure represents a notable surge compared to the range of cases reported in 2015, which was between 9 and 26 million [2, 3].

The physiopathology of diabetic foot infections (DFI) is complex, but its severity and prevalence result from host-related disorders and pathogens-factors, such as virulence and antibiotic resistance traits [2]. Several studies have shown that DFUs are classically polymicrobial-infected [3], characterized by several pathological sequelae, such as neuropathy and peripheral vascular disease [4, 5]. 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 touches the surface of an ulcer is likely to colonize it. Medical personnel, supplies, and drugs used for therapy frequently unwittingly spread harmful microflora. Various bacteria strains are often present in the mixed flora of ulcers [6, 7]. Staphylococcus aureus (S. aureus) is frequently identified as the predominant pathogen in these cases. Infections in these ulcers are characterized by disruptions in extracellular matrix remodeling, abnormal angiogenesis-related growth factors, and excessive inflammatory responses, all hindering wound healing [8]. However, if S. aureus bacteria develop antibiotic resistance, they can lead to severe opportunistic infections or diseases. According to a US Centers for Disease Control and Prevention (CDC) survey, approximately 5% of the population carries the MRSA strain [9]. Diabetic foot ulcers (DFUs) caused by antibiotic-resistant bacteria, particularly Methicillin-Resistant Staphylococcus aureus (MRSA), are associated with more severe infections [10]. In addition to producing a slim layer, *S. aureus* is involved in the infection of soft tissues and bones. The slim layer (glycocalyx or biofilm) forms bacteria that infect foot ulceration, which is the cause of 80% of lower-limb amputations. Toxins, which can cause tissue necrosis, progression, and spread of infection in DFI patients, can also be released by *S. aureus* in addition to basic adhesion mechanisms [10-12]. Methicillin-resistant *S. aureus* (MRSA), which accounts for most *S. aureus* infections, is present in 10-40% of diabetic wounds and produces Panton-valentine leukocidin (PVL). This cytotoxin is crucial to methicillin-resistant *Staphylococcus aureus* virulence [13, 14].

MRSA was initially identified in England 1961, shortly after introducing methicillin, the first penicillinase-resistant semi-synthetic penicillin [15]. Since then, MRSA has emerged as the predominant pathogen responsible for hospital-acquired infections worldwide, with its incidence still on the rise in many countries [16]. MRSA exhibits resistance to multiple antibiotics, including methicillin, penicillin, oxacillin, cloxacillin, cefazolin, cefoxitin, and other commonly used antibiotics [17]. Transmission of MRSA can occur through close contact with infected individuals or via contaminated objects. In healthcare settings, infections acquired in hospitals or other healthcare facilities are called nosocomial infections [9].

Once Staphylococcus aureus adheres to host tissues, it can create biofilms. These biofilms facilitate its persistence by allowing bacteria to evade host defenses, obstructing access to certain immune cells like macrophages. These immune cells struggle to penetrate the biofilm matrix fully, leading to a phenomenon known as frustrated phagocytosis [2]. Additionally, cells within biofilms exhibit heightened antibiotic tolerance, making it challenging to treat S. aureus infections that have formed biofilms. The fibronectin-binding proteins (FnBPs), including FnBPA and FnBPB, play a role in stimulating biofilm formation by clinically relevant MRSA strains [18].

In contrast to heritable antibiotic resistance mechanisms, biofilm-associated tolerance represents a temporary state wherein bacteria typically susceptible to antibiotics undergo physiological changes that reduce their sensitivity. When these cells disperse and return to a planktonic state, they regain their normal susceptibility profile [19]. Bacteria entrenched within biofilms pose a challenge for eradication due to various nutrient gradients that hinder or halt bacterial growth, protein synthesis, and other physiological activities. Bacteria within biofilms are less responsive to antibiotics due to their reduced growth rates [20]. Additional factors contributing to biofilm-mediated antimicrobial resistance include inefficient diffusion or entrapment of the antibiotic within the biofilm matrix, the presence of "persister" cells, and other unidentified phenotypic differences [20].

This study aimed to identify methicillin-resistant *Staphylococcus aureus* genetically from DFU patients and discuss its function in Biofilm formation. It also identified the genes that are the primary contributors to delayed healing and lower limb amputations, produced the cytotoxin (pvl), the primary cause of tissue necrosis, and assessed the frequency of its genes in MRSA isolated from infected DFU patients.

Materials and Methods

Morphological and Biochemical Characteristics

A total of 80 swabs from the necrotic lesions of patients with 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 mellitus (T2DM), which had an average duration of 14.5±2.5 years. The patients were between 40 and 55, plus or minus five years. These swabs were cultured in sterile tubes with 5ml of Brain Heart Infusion Broth-BHIB (HIMEDIA; India) and then moved to the laboratory, incubated for 24h at 37°C. The Staphylococcus Chromogeneic Agar Media (CONDA Pronadisa; Spain) was streaked after the broth media showed positive growth. Colonies developed after being cultivated on nutrient agar (HIMEDIA; India) for 24 hours at 37°C [21]**.** Gram staining was done to identify the colonies.

Congo Red Agar (CRA) method

According to Freeman [22], this agar was made by mixing blood agar base with 0.8gm congo red, 50gm sucrose, and 1-liter distilled water. The pH was then adjusted to 8, and the agar was autoclaved at 121C for 15 minutes.

Bacteria 16srDNA Genotyping

Extraction of DNA

The DNA extraction was done using a genomic DNA micro kit (Geneaid; Taiwan).

I6srDNA amplification and sequencing

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 27F (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 [23]. The positive samples of PCR products for the universal *16srDNA* gene were sent to Bioneer Company (Korea) for sequencing to identify them further.

16srDNA identification

The 16srDNA sequences of the bacterium isolates were compared to the Genbank 16srDNA 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 [24] and Luk-PV-2, depending on [25]. The slime layer is encoded by the icaA gene, which has the primers icaA1 and icaA2, according to [25]. The pvl genes use the primers Luk-PV-1 and Luk-PV-2 to detect the PVL S/F bicomponent proteins, depending on [26]**.** *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.

Findings

Morphological and Biochemical Characteristics

Staphylococcus aureus was isolated and found to be dependent on its color (pink to mauve) on Staphylococcus Chromogenic agar media.

Table 1. Alignments of PCR product for16SrDNA gene (all 100% identical with reference strain)

Sequence	Bacterial strain
***Staphylococcus aureus* strain pak2 (706 bp, n=5)**	CTGTGCAATCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG CGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAG GAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGC GAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGC TAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAAT TCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATG GTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGTTGA CTGCCGGTGACAAAC
***Staphylococcus aureus* strain MR30 (660bp, n=3)**	GGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTA GGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCA TGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATC AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACT CCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG AACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGT GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTT
***Staphylococcus aureus* strain CFSAN007896 (706bp, n=3)**	AGTCCAGTAACTGTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCG GAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGA GTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGA CGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTT AGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCA TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACA GGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCA CTCTAAGTTGACTGCCGGTGCTACCAAAAAAAAAAAAAAAAAAAAAAAAAAAATATGTATAAA
***Staphylococcus aureus* strain ST4 (676bp, n=1)**	TCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGC GCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGG AATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTG GGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTA AGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC GCGAAGAACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCA GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCT

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.

I6srDNA 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 1500bp 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 (Table 1), which was the same strain. These include the *S. aureus* strains pak2 (n=5), MR30 (n=3), CFSAN007896 (n=3), and ST4 (n=1).

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

Amplification of the *mecA* and *icaA* genes produced bands at 310bp and 188bp, respectively, as shown in Figure 1.

Nine (75%) of the 12 isolates encode for the *pvl S/F* gene (Bicomponent proteins), which produced bands at position 433bp.

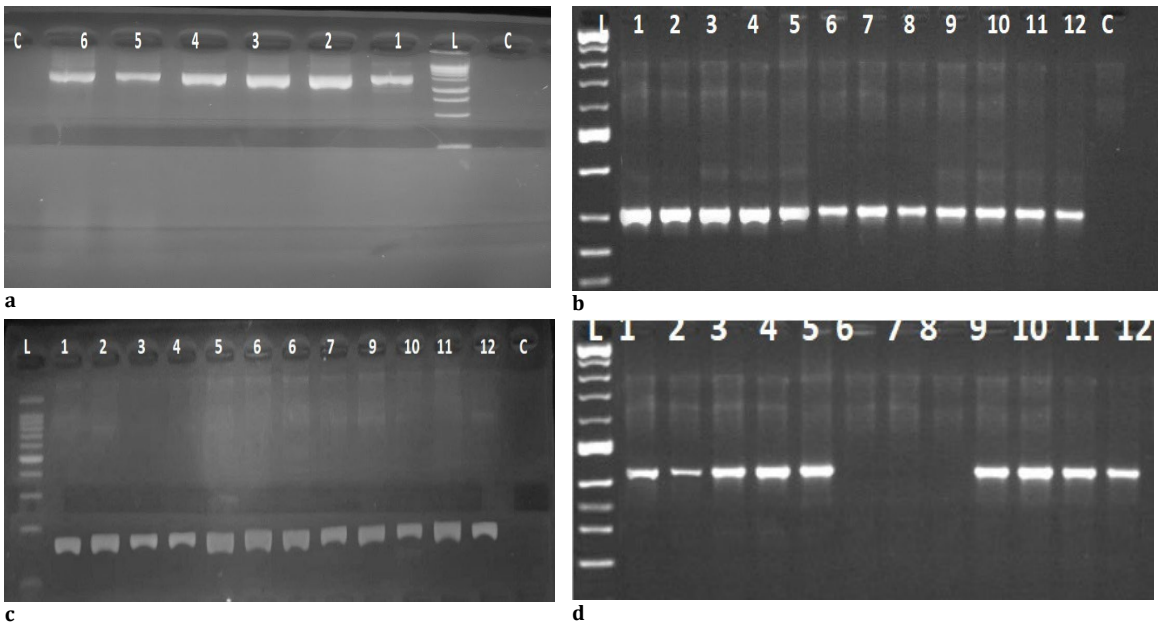

Figure 1. (a) Electrophoresis of agarose gel (1%) for Universal *16srDNA* PCR products at position 1500bp from (1-6), L: (Ladder:250-10000bp); (b) Electrophoresis of agarose gel (1%) from (1-12) specific mecA gene products at position 310bp, L: (Ladder:100-1000bp); (c) Electrophoresis of agarose gel (1%) from (1-12) specific icaA gene products at position 188bp, L: (Ladder:100-1000bp); (d) Electrophoresis of agarose gel (1%) from (1-9) specific *pvl S/F* gene products at position 433bp, L: (Ladder:100-1000bp)

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 [27, 28]. The collected DNA was amplified with the help of universal 16srDNA primers (F27 and R1492). The primers avoid the loss of any potential or new bacterial strains by amplifying the *16srDNA* (16s ribosomal DNA) gene for all bacterial strains [28-29]. 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 [30] and Bandar Abbas, southern Iran [31]. Additionally, this study's prevalence of MRSA was 100% of *mecA* in locations where methicillin-resistant *S. aureus* 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%) [32, 33].

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 incorrect sterilizers to treat ulcerated wounds. Any diabetic foot infection treated with MRSA will likely lead to increased resistance and 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 are necessary for S. aureus to be harmful, and 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 forming 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 inhibiting phagocyte death after ingestion [7, 34, 35].

In addition to biofilm formation, *S. aureus* contains a wide range of cytotoxins, which significantly impact the progression and spread of the bacteria in DFUs and potentially result in tissue necrosis [36]. In this investigation, most isolates from DFU patients have icaA and pvl genes. This outcome aligns with research done in France and Iraq [12, 37].

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 the 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.

Conclusion

Panton-valentine leukocidin has a high prevalence among MRSA strains isolated from diabetic foot ulcer patients that form biofilms, causing patients to have significant inflammation, illness progression, and challenging wound healing, which may result in lower limb amputations.

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