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Original Research Paper

Molecular identification of *Staphylococcus aureus* strains isolated from diabetic foot ulcers based on 16SrRNA

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Abstract: *Diabetic foot ulcer (DFU) is the most serious diabetic complication. Gangrene is caused by successive bacterial infections invading diabetic wounds and may lead to limb amputation for the diabetic patient. Staphylococcus aureus (S. aureus) is one of the most prevalent bacterial pathogens recovered from diabetic foot infections (DFIs). It is found worldwide and is a leading cause of disease. Many methods have been used for identifying the associated strains in clinical specimens. Sequencing by 16SrRNA methods is achieved in the identification. The current study aimed to detect s aureus isolated from Diabetic foot ulcers by 16SrRNA. One hundred clinical samples were collected from patients with diabetic foot ulcers at Faida Primary Health Center, Mosul City, Iraq. The bacterium was identified using Gram stain, colony morphology, biochemical tests, selective differential media, and molecular analyses. A total of 60 S. aureus isolates were obtained out of 100. In the molecular study, 10 isolates from 60 were selected for PCR assay. Selected isolates were confirmed by PCR assay that successfully produced amplified product 16SrRNA gene with size 164 bp. Our results support the recent view that gram-positive organisms, depending on the geographical location, may be predominantly DFIs.*

Keywords: *Diabetes; ulcer; PCR; Staphylococcus aureus; 16SrRNA.*

1.Introduction

A chronic metabolic condition called diabetes mellitus is defined by blood glucose levels that are higher than usual. Over time, this disorder causes significant harm to several body organs, such as the kidneys, heart, blood vessels, eyes, and nerves [1].The emergence of infected wounds is one of the most dreaded problems that people with diabetes face. Wound healing is frequently delayed in diabetics due to hyperglycemia, vasculopathy, neuropathy, and immunocompromised conditions [1]. The World Health Organization (WHO) estimates that 108 million individuals worldwide had diabetes mellitus in 1980, although the figure is continuously increasing. From 151 million in 2000 to 194 million in 2003, 246 million in 2006, 285 million in 2009, 366 million in 2011, and 425 million in 2015, there were more diabetes people worldwide [1]. It is projected that 643 million people worldwide will have diabetes by 2030, and 783 million by 2045 [2]. It is believed that 10–15% of people with diabetes have diabetic foot ulcers (DFUs). When these ulcers are first discovered, almost half of them are determined to be clinically infectious. In these instances, beta-hemolytic *Streptococci* and *Staphylococcus aureus* (*S. aureus*) are the most common causes of skin infections. Conversely, people from low-resource environments are more likely to have gram-negative bacteria, such *Pseudomonas aeruginosa (P. aeruginosa*),

found in their bodies. Furthermore, it is recognized that fungal infections may present a challenge in the treatment of diabetic wounds [3]. A 2015 research by the International Diabetes Federation found that over 415 million people worldwide suffer from diabetes (5 million of them pass away annually) and that by 2040, the incidence of the disease is predicted to reach over 640 million individuals (1 in 10) worldwide. (IDF 2015) [4]. Estimates show that someone with diabetes loses a lower limb to the condition every 20 seconds. When there are purulent discharges or at least two of the common signs of inflammation-pain or tenderness, warmth, redness, swelling-in a diabetic foot wound (pus), it is considered an infection [5]. Foot problems now account for more hospital stays for diabetic patients than any other type of condition. One important aspect of diabetes is diabetic foot ulcers (DFU). Skin ulcers known as DFUs often progress down the legs and are accompanied by peripheral neuropathy and vasculopathy. Compared to other devices, DFUs were found to have higher rates of morbidity, sickness, death, and psychological costs in several investigations. Osteomyelitis and gangrene can result from DFU. To cure severe DFU, a considerable limb is frequently amputated [6]. For the therapy of DFU, a variety of abilities, competencies, and experiences are needed. Patient education and better care classification are crucial in preventing amputations [7]. To learn more about DFU microbiota, new diagnostic techniques such as the 16S ribosomal DNA sequence in bacteria should be applied. DFU's polymicrobial characteristics include individualized antimicrobial-driven treatment, debridement, wound evaluation, and dressing changes [8]. The following characteristics are often helped by new biological and molecular medications that enhance local inflammation management, infection prevention, and cicatrizing mechanism efficiency. Some of the most recent developments in antimicrobial therapy include devices, ozone, energy-based therapies, plant extracts, antimicrobial peptides, cellular, gene, and molecular treatments [1]. The pathogenesis of DFUs is multifaceted and includes trauma, PAD, and diabetic neuropathy. Before and following their appearance as a halt in wound healing, both variables have a role in the formation of ulcers [1]. Due to certain immunological characteristics, DFU healing is delayed in diabetics. Among these reactions include T-lymphocyte apoptosis, elevated pro-inflammatory cytokines, and impairment of polymorphonuclear cell activities such as chemotaxis, adhesion, phagocytosis, and intracellular killing. Among these effects include suppression of fibroblast growth, damage to keratinocytes' basal layer, and reduced migration of epidermal cells [9]. High blood glucose promotes the growth of bacteria, particularly aerobic Gram-positive cocci such as *S. aureus* and hemolytic *Streptococci* [1]. Skin, nares, and mucosal surfaces are only a few of the areas of the human body where *S. aureus* colonizes. It is a common, Gram-positive commensal pathogen [10]. *S. aureus* colonizes around one-third of healthy individuals [11]. *S. aureus* is an opportunistic pathogen that may express and acquire a wide range of virulence factors and antibiotic resistance determinants in both the community and hospital. As a result, it can result in a wide range of nosocomial and community-acquired infections in people all over the world, from minor illnesses to fatal ones [11, 12]. To get a deeper understanding of the pathophysiology, epidemiology, and treatment resistance of *S. aureus*, comparative genomics analysis which includes transcriptome and proteome levels, and sequence comparison has become more and more popular [13]. To investigate the genetic linkages and relatedness of S. aureus strains, as well as to provide light on the evolution of *S. aureus*, a range of molecular typing approaches have been established for *S. aureus* isolates [13]. To identify *Staphylococcus* species, the 16S RNA gene sequencing and PCR restriction fragment length polymorphism study were classified [14]. Although phenotypic methods have traditionally been used by clinical microbiology labs and public health organizations to categorize bacterial infections, conventional culture was long regarded as the gold standard for bacterial identification. However, certain therapeutically significant bacteria grow slowly, are difficult to culture, are fussy, or are frequently uncultivable, making it necessary to evolve germs effectively over the course of days or even weeks [15]. In a microbiome analysis of fresh and chronic DFUs, a 16SrRNA amplicon sequencing analysis revealed that *S. aureus* was the most frequently isolated Gram-positive bacterium, followed by *Escherichia coli*, *Proteus* spp., *Enterobacter* spp., and *Citrobacter* spp., and *Anaerococcus* were the most frequently isolated Gram-negative bacterial species, respectively. The origin of DFUs is significantly influenced by geography [16]. Therefore, the primary goal of the current study was to use the PCR method to identify *S. aureus* isolated from diabetic foot ulcers using the 16S RNA gene.

2.Methodology

Bacterial isolation

The study included 100 clinical samples taken from diabetic adult patients with diabetic foot ulcer (DFU) by an expert physician with their consent at Faida Primary Health Center, Mosul City, Iraq from November 2023 to February 2024. Sampled patients included both genders and different ages. The samples were transported to the Microbiology Laboratory at the Department of Basic Sciences, College of Nursing

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within 1-2 hours for culture and bacteriological detection.

Bacterial identification

All isolates were first cultured using sterile cotton swabs in sterile vials containing Nutrient broth that was incubated at 37°C/24 hr for microbiological identifications. Each sample was then inoculated onto Mannitol salt agar to isolate *S. aureus*. Bacterial identification was done by using Gram stain, and biochemical tests (catalase test, coagulase test, oxidase test, and IMViC test). The confirmation test done was by using the Automated Vitek 2 system (bioMérieux, Lyon, France).

Molecular identification of Staphylococcus species using 16SrRNA

Extraction of DNA

For DNA extraction, the boiling method was performed, by taking two to three fresh colonies from a bacterial plate and mixing them in 800 µl of deionized water. Then placed in a water bath at 100°C for 10 min followed by centrifugation at 1200 rpm for 2 minutes using a micro centrifuge (Mini Spin Plus, Eppendorf). Crude lysate containing DNA was stored at -20°C for use over a long period [17].

Determination of DNA Concentration and Purity

The concentration and purity of the DNA of each isolate were determined using a Nanodrop machine (Molecular Department, General Central Laboratory, General Health Directorate in Duhok). Confirmation of the quantity and purity of the DNA was calculated by the Nanodrop 2000 spectrophotometer (Thermo Scientific Germany). The purity of the DNA was judged based on an optical density ratio of 1.7-1.8 and a concentration between 10-50 nanog /µl [17].

Conventional Polymerase Chain Reaction (PCR)

The DNA of S. aureus isolates was targeted for the 16S rRNA gene using the primer listed in (Table 1). A reaction mixture (20 µl) contained 3 µl of DNA, 1 µl of each primer, 10 µl of Master Mix, and 5 µl of nuclease-free water [18]. The conventional PCR machine is set up with many cycles and different temperatures for each step, including step one, Initial denaturation. Followed by step two (denaturation, annealing, and Extension), and finally an extra extension. PCR program for the 16SrRNA gene is summarized in (Table 2), then the PCR products were analyzed using 1.5% agarose gel electrophoresis, and the ethidium bromide-stained bands in the gel were visualized using a Gel imaging system [17].

Table 1: Primer sequences used for PCR

Gene	Primer sequence (5'-3')	Product size (bp)	Reference
16S rRNA-F	GGTCTTGCTGTCACTTATAGATGG	164	[18]
16S rRNA-R	CGGAAGATTCCCTACTGCTG		

Table 2: Steps of thermal cycling, (PCR program) for gene 16SrRNA

Steps	Temperature °C	Time m:s	Cycle
Initial denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	60	00:30	
Extension	72	00:30	
Final extension	72	00:30	1
Hold	10	10:00	

Statistical analysis

All data statistical analysis was done using SPSS version 20 (IBM USA).

3. Results and discussion

The present study focused exclusively on *S. aureus* isolated from patients with diabetic foot Infections. *S. aureus* is associated with significant increases in hospitalization, infections, and treatment for patients, and morbidity and mortality rates among patients with diabetic foot.

The samples were taken from the diabetic patient's wound using a sterile swab, as shown in (Figure 1), under the cautious observation of a specialist physician in an aseptic setting. The information shown in (Table 3) is based on 100 clinical samples. While 40 (40%) of the samples were negative and 60 (60%) of the samples revealed positive isolates for *S. aureus*, (Table 4) listed the patient's age and the bacterial cultures found after microscopic inspection. The information showed that patients with diabetic wounds between the ages of 50 and 59 were most vulnerable to bacterial contamination and pathogenicity.

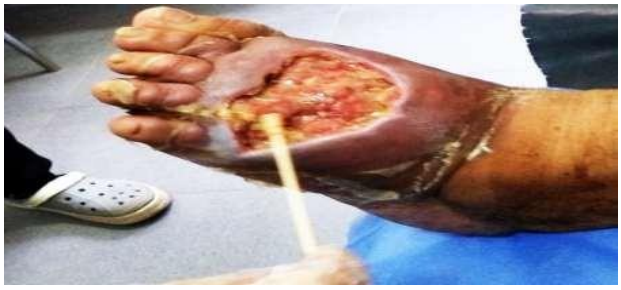

Fig 1: Isolation of pathogenic bacteria from diabetic wound using swab method

Table.3: Number and percentage of *S. aureus isolates*.

Number of samples	Positive	Negative
100 (100%)	60 (60%)	40 (40%)

Table 4: Number and percentage of bacterial isolates according to patients' ages

Age (years)	No. & % of +ve S. aureus isolates
30-39	10 (16.7%)
40-49	15 (25%)
50-59	27 (45%)
60-69	8 (13.3%)
Total	60 (100%)

Ten *S. aureus* isolates were chosen for PCR analysis after being examined out of 60 isolates for molecular detection.

All ten of the chosen isolates were proven to be *S. au reus* by phenotypic analysis and PCR, which effective ly produced an amplified product that targeted the 16 S rRNA gene (Figure 2).

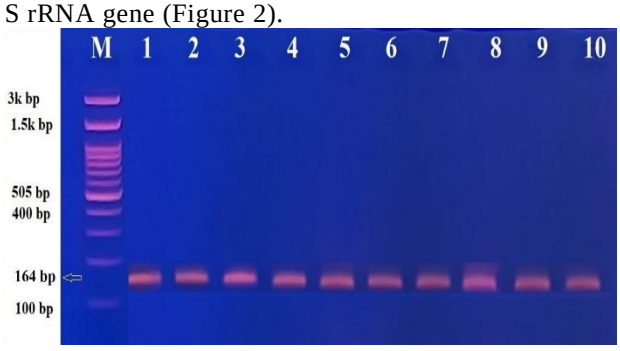

Fig 2: Represents 1.5 % agarose gel showing amplification product of 16S rRNA gene (164 bp); lanes 1-10 showing represent positive *S. aureus* isolates.

One of the most prominent effects of diabetes is diabetic foot infection (DFI), which is brought on by one or more bacteria [19, 20]. This study's findings are consistent with those of a prior investigation conducted in Egypt by Saleh and colleagues, which showed that 62/100 (62%) of *S. aureus* isolates were identified out of 100 clinical samples that were obtained and collected from patients [21, 22]. According to this study, people with diabetes between the ages of 50 and 59 had the highest risk of wound contamination and infection. These findings concur with recently released research on the relationship between patient age and DFU distribution [23, 24]. A potent method for the detection and identification of bacteria is the amplified DNA from phylogenetically diverse bacteria that targets conserved sections of the 16S rRNA gene. The results of this study indicated that 16S rRNA gene PCR was a highly effective technique for identifying *S. aureus* isolates. These findings concur with those of earlier research that have shown that identifying clinical isolates of *S. aureus* using the detection and sequencing of this gene is a useful method [14, 18, 25]. A gene known as 16S rRNA has shown itself to be a highly conserved molecular target in populations across time [27]. Therefore, in clinical microbiology labs, 16S rRNA sequencing has been crucial to the precise identification of bacterial isolates and the discovery of new bacteria [28].

Conclusion

Numerous microorganisms can cause diabetes-related foot ulcers. The total prevalence of *S. aureus* bacteria increased in this research, which underscored the need for microbiological investigation prior to starting therapy for diabetic foot ulcer infections. Additionally, as the 16S rRNA result provided a clear image of the identification of *S. aureus* bacteria from clinical samples, our study validated the importance of 16S rRNA in the molecular detection of *S. aureus*.

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Conflict of interests:

No conflict of interest is declared.

Author's Contributions

Both authors designed the study, collected data, analyzed the results, and drafted the manuscript; as well as read and approved the final version of the manuscript.

Ethics

The study protocol was approved by the arch Ethics Committee / Scientific Research Division / Directorate of Planning / Duhok Directorate General of Health / Ministry of Health / Kurdistan Regional Government / Iraq [2024].

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