Original Article

Molecular Detection of Some Virulence and Antibiotic Resistance Genes of *Staphylococcus aureus* Isolated from Diabetic Foot Infection

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Abstract

Background: Diabetes mellitus is a chronic disease that continues to increase meaningfully. One of its significant complications is foot infections. *Staphylococcus aureus* is one of the predominant isolates in diabetic foot infections. **Objectives:** Determination of the severity of biofilm-forming bacteria that infect diabetic foot infections in Kerbela city by isolation of these species and diagnosis of them molecularly. **Materials and Methods:** In this study, *Staphylococci* isolated (n = 25) from diabetic foot were identified, susceptibility test against 17 traditional antibiotics, quantification of biofilm formation was performed, screened for virulence characters by polymerase chain reaction (PCR) technique was applied for detection of five genes. **Results:** Among the 119 isolates obtained in this study, 25 *S. aureus* were identified. All the isolated *S. aureus* was most sensitive (84%) to levofloxacin, whereas the lowest sensitivity (36%) was toward azithromycin and erythromycin, 40% of the sensitivity was toward penicillin, amoxicillin–clavulanic acid, ceftazidime, cefepime cefotaxim, ceftriaxone, imipenem, meropenem, aztreonam, and oxacillin, and the sensitivity of both rifampin and vancomycin was 52%, and ciprofloxacin was 48%. According to the microtiter plate method, 60% of *S. aureus* obtained from diabetic foot infection (DFI) in the current study were strong producers of biofilm, whereas 40% of them were moderate. PCR technique detected that 60% of *S. aureus* contained the *mec*A gene and 64% contained *ica*A, *sea*, and *erm*C genes. Finally, 80% contained the *ica*D gene. **Conclusions:**This study displays that diabetic foot ulcer *Staphylococci* present resistance to antibiotics and shelter for virulence determinants. These properties propose *Staphylococci* can participate in the perseverance and severity of these infections, leading to treatment disappointment.

Keywords: Antibiotic resistance, biofilm, diabetic foot infection, MRSA, virulence factors

Introduction

Foot ulcers are a growing problem in patients with diabetes mellitus and infection is a common complication that essentially establishes the greatest common reason for hospitalization in diabetic patients, frequently associated with lower-extremity amputation.[1] Some studies have confirmed that they characterize an economic problem worldwide, equivalent to the costs accompanying cancer, depression, lung, and musculoskeletal diseases.[2] In DFI, the patterns of microbial infection are not constant. The bacterial profile revealed a polymicrobial pattern and *Staphylococcus aureus* was the most frequent microorganism isolated as shown in a study done in Tanzania by Chalya *et al.*[3] *Staphylococcus* is a common commensal bacteria of human skin and mucosa, is one of the chief causes of infections in humans, extending from minor skin infections to severe infections, such as septicemia, endocarditis, and osteomyelitis.[4] These bacteria can produce numerous virulence factors, one of the most essential being biofilm formation, which is made up of adherent bacterial populations growing inside their polymeric structures that provide the capability of

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evasion to the immune system and multiple antibiotic treatments.[5] It has become progressively more common for methicillin-resistant *S. aureus* (MRSA) to spread among foot ulcer patients over time, which frequently results in amputations.[6]

Materials and Methods

Study design and sample collection

This study was designed as a cross-sectional study, whereas 142 swab samples were collected from patients checked at Imam Al-Hasan Center for Endocrinology and Diabetes from October 2022 to January 2023. Samples were collected from 142 patients with diverse grades of wounds/ulcers and gangrene. Before sample collection, the DFU was cleaned with a sterile saline solution. Samples were collected by using a surface swab with transport media to conserve bacteria until cultivation on suitable media. Different types of media were used for sample immunizations; that is, blood, MacConkey, and mannitol-salt purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India. All plates were incubated for a maximum of 5 days or until visible growth using aerobic conditions at 37°C. Cultures with any bacterial growth were informed as laboratory positive.

Bacterial isolation

The specimens were inoculated onto blood agar and MacConkey's agar purchased from HiMedia Laboratories Pvt. Ltd. The inoculated plates were incubated at 37°C overnight, and the plates were examined for growth, the next day. Further processing was done according to the nature of the isolate, as was determined by Gram staining and the colony morphology. The organisms were identified based on their Gram-staining properties and their biochemical reactions. Bacterial isolates were recognized by standard and biochemical methods, such as potassium hydroxide test, catalase production, coagulase, oxidase, and mannitol fermentation. In addition, the diagnosis of *S. aureus* by using API STAPH Kits purchased from bioMérieux, Craponne, France, was performed.

Antibiotic susceptibility

Disc diffusion method was performed according to Hudzick[7] as determined by the Clinical and Laboratory Standards Institute guidelines to check the susceptibility of the most prevalent bacteria to the most traditional antibiotics manufactured by Liofilchem, Roseto degli Abruzzi, Italy.

Investigation of biofilm formation

Quantification of Biofilm formation by microtiter plate method

The method described by Kırmusaoğlu[8] was performed with some modifications to investigate the ability of bacteria to form a biofilm, whereas the young isolates were inoculated into 5 mL of a Brain Heart Infusion Broth and were incubated at 37°C for 24 h, and the bacteria were diluted with the same media and compared with a 0.5 standard McFarland solution then 200 µL of diluted bacterial culture were transferred into every well in microtiter plate in four duplicates for each isolate. Uncultured Brain Heart Infusion Broth was added as a control into the wells then the microplate was incubated at 37°C for 24 h after closing it tightly after that the culture was drained, and the wells were washed three times by normal saline then the microplate was dried in an oven at 60°C for 30 min and 200 µL of 0.5% crystal violet were added and were left for 15 min also the stain was drained and the wells were washed three times until the dye was disappeared then they were let to dry then 200 µL of 33% of glacial acetic acid were added. In addition, the optical density was measured at 630 nm by an enzyme-linked immunosorbent assay reader. Finally, the efficiency of isolates to form the biofilm was determined by comparing the optical density as described below.

Mean OD value	Biofilm formation
$OD \leq ODc$	None
$ODc < OD \leq 2 \times ODc$	Weak
$2 \times ODc < OD \leq 4 \times ODc$	Moderate
$4 \times ODc < OD$	Strong

Molecular detection of virulence and antibiotic resistance genes by PCR

Total deoxyribonucleic acid (DNA) was extracted using a DNA extraction kit purchased from ADDBIO, Yuseong, Daejeon, Korea. The resistance and virulence genes, including *mec*A, *ica*A, *ica*D, *sea*, and *erm*C were distinguished by the polymerase chain reaction (PCR) technique using specific oligonucleotide primers [Table 1]. PCR reactions were performed in the PCR Thermal Cycler (Labnet International, Edison, NJ, USA) using PCR Master Mix (Microgen, Jinju, South Korea). PCR reactions were performed in total volumes of 23 µL. The master mix contained 10 µL of the reaction mixture with Taq DNA polymerase master mix, 1.5 µL of each of the forward and reverse primers in 10 µM/µL concentrations,3 µL of target DNA, and 7 µL of distilled water. The PCR mixtures were subjected to the PCR reaction conditions, which included one cycle for initial denaturation of templet DNA for 4 min at 94°C for *mec*A gene, 5 min at 95°C for *ica*A and *ica*D genes, 5 min at 94°C for *sea* gene, and 10 min at 94°C for *erm*C gene. While the second step included 30 cycles involving denaturation of templet DNA for 45 s at 94°C for the *mec*A gene, 2 min at 95°C for *ica*A and *ica*D genes, 2 min at 94°C for the *sea* gene, and 30 s at 94°C for e*rm*C gene followed by annealing of primers with template DNA for 45 s at 50°C for *mec*A gene, 20 s at 60°C for *ica*A and *ica*D genes, 2 min at 57°C for *sea* gene, and 30 s at 53°C for *erm*C gene, whereas the extension involving 1 min for *mec*A, *sea*, and *erm*C genes and 20 s for *ica*A and

Table 1: Primers with their sequences and amplicon sizes of *Staphylococcus aureus*

Gene	Primers	Primer sequence 5′–3′	Product size (bp)	References
*mec*A	*mec*A–F	GTAGAAATGACTGAACGTCCGATAA	310	Geha *et al.* (1994) [9]
	*mec*A–R	CCAATTCCACATTGTTTCGGTCTAA		
*ica*A	*ica*A–F	GAGGTAAAGCCAACGCACTC	151	Mahmoudi *et al.* (2019).[10]
	*ica*A–R	CCTGTAACCGCACCAAGTTT		
*ica*D	*ica*D–F	ACCCAACGCTAAAATCATCG	211	Mahmoudi *et al.* (2019).[10]
	*ica*D–R	GCGAAAATGCCCATAGTTTC		
sea	*sea*-F	GGTTATCAATGTGCGGGTGG	102	Mehrotra *et al.* (2000) [11]
	sea-R	CGGCACTTTTTTCTCTTCGG		
*erm*C	*erm*C–F	GCTAATATTGTTTAAATCGTCAATTCC	572	Ghanbari *et al.* (2016) [12]
	ermC–R	GGATCAGGAA AAGGACATTT TAC		

*ica*D genes at 72°C. Lastly, the final extension required 2 min for *mec*A, 5 min for *ica*A and *ica*D genes, 7 min for the *sea* gene, and 10 min for *erm*C gene at 72°C. The PCR products were exposed by electrophoresis on a 1.5% agarose gel using 70 V for 50 min followed by consequent exposure to ultraviolet light in the presence of DNA load dye.

Statistical analysis

Data were analyzed by the Statistical Package for the Social Sciences (version 19) software. Descriptive statistics were used as mean and percentage, and the chi-square test was used for analysis. A *P* value of less than 0.05 was considered significant.

Inclusion and exclusion criteria

Patients with type 2 diabetic foot ulcer were included in this study irrespective of the stage of ulcer infection, and the age of subjects was ≥35 years old. However, patients aged <35 years, non-diabetic patients with foot ulcers, type 1 diabetic mellitus, and pregnant women were excluded.

Ethical approval

This study was approved by the Ethical Committee at the College of Science, University of Karbala, Karbala, Iraq. All subjects involved in this work were informed and agreement was obtained verbally from each one before the collection, whereas 142 swab samples were collected from patients checked at Imam Al-Hasan Center for Endocrinology and Diabetes from October 2022 to January 2023.

Results

Of the total 142 diabetic foot patients studied 84 were males and 58 were females. Their ages ranged from 35 to ≥75 years. The maximum number of patients having diabetic foot infections was in the age group of 55–64 years. Among the 142 samples, 23 exhibited no growth, whereas 119 isolates were positive cultures, of these, 82% were polymicrobial, and 18% were monomicrobial. Overall, 119 isolates were collected from 142 cultures. Among the 119 isolates 63 (44%) isolates of Gram-positive and 56 (39.4%) of Gram-negative bacteria. Particularly, of Gram-positive bacteria, *S. aureus* was the most prevalent as 25 (21%) isolates, *Staphylococcus epidermidis* 18 (15%), *Streptococcus agalactiae* 15 (13%), and *Enterococcus faecalis* 5 (4%), whereas of Gram-negative, *Klebsiella pneumoniae* has the greatest frequency as 25(21%) isolates, *Pseudomonas aeruginosa* 13 (11%), *Proteus mirabilis* 10 (8%), and *Escherichia coli* 8 (7%) as mentioned in Figure 1.

Susceptibility test demonstrated that all isolated *S. aureus* was most sensitive (84%) to levofloxacin, whereas the lowest sensitivity (36%) was toward azithromycin and erythromycin, 40% sensitivity was toward penicillin, amoxicillin–clavulanic acid, ceftazidime, cefepime, cefotaxime, ceftriaxone, imipenem, meropenem, aztreonam, and oxacillin, and the sensitivity of both rifampin and vancomycin was 52%, and ciprofloxacin was 48%.

Investigation of biofilm by tube method illustrated that all of the 25 isolates of *S. aureus* obtained from DFI were biofilm formers compared with control tube, whereas according to the microtiter plate method, 60% of *S. aureus* obtained from DFI in the current study were strong producers of biofilm, and 40% of them were moderate producers.

In this study, the PCR technique was used to detect the presence of the *mec*A gene in *S. aureus*, which indicates that *S. aureus* is methicillin-resistant. Figure 2 shows the electrophoresis of the PCR products, through which it is clear that the primer of the *mec*A gene was successful in amplifying this gene through the appearance of a PCR product of 310 bp in size. PCR technique was also used to detect the presence of the *ica*A gene, which is responsible for biofilm formation. Figure 3 shows that *ica*A primer was successful in amplifying this gene through the appearance of a PCR product of 151 bp in size in 64% of *S. aureus* isolates. The presence of the *ica*D gene, which is responsible for biofilm formation was detected. Figure 4 shows the *ica*D gene was successfully amplified, which has been indicated by the presence of a PCR product size of 211bp in 80% of *S. aureus*. The current study included an

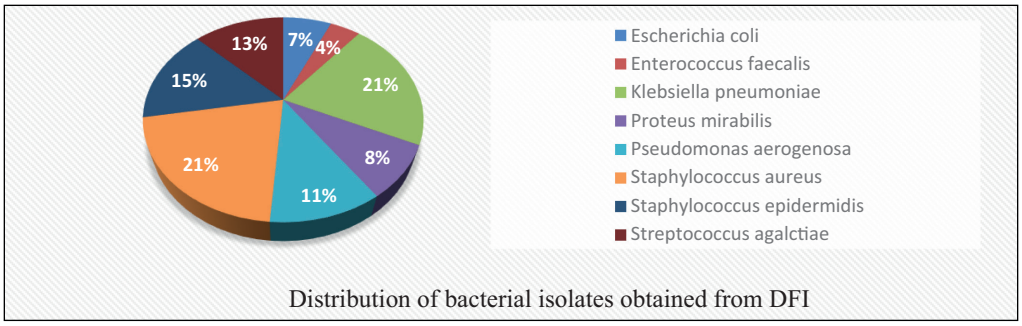

Figure 1: Distribution of bacterial isolates obtained from diabetic foot infection

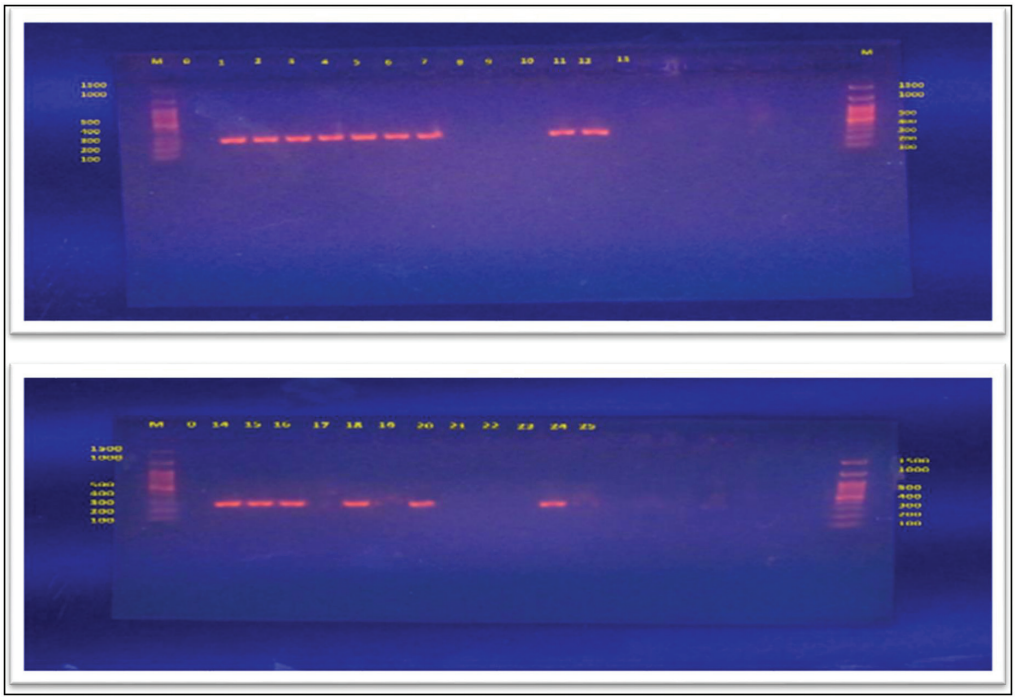

Figure 2: Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *mec*A gene (310 bp) using 1.5% agarose gel, 70 V for 50 min

investigation presence of the *sea* gene, which is responsible for the production of enterotoxin. The PCR technique in Figure 5 displays the successful amplification that has been distinguished by the appearance of bands related to *sea* gene PCR product size of 102bp in 64% of *S. aureus* isolates. Finally, the presence of the *erm*C gene was detected. Figure 6 shows the successful amplification of the *erm*C gene product size of 572bp in 64% of *S. aureus* isolates obtained from DFI in the current study.

Discussion

In the current study, with the exclusion of no growth swabs, males were the most vulnerable to diabetic foot infection (62.18%) compared with females (37.81%). These results approach Aleem *et al.*[13] findings, which documented that males and females formed (66% and 34%), respectively, of total diabetic foot infection patients in India.[13] Higher male incidence may be due to the higher level of outdoor physical activity with insufficient and inappropriate foot care among males in comparison with females. This result might probably have been credited to the higher level of outdoor activity, better admission to health care skills, deficiency of foot care, and poor acquiescence to foot care tradition as compared to females. Additionally, male prevalence in DFU could be related to several factors such as gender-related variation in lifestyles, inadequate hygiene, kind of footwear, and professional jobs that impose the feet to tolerate more pressure as a result of work and make them more exposed to trauma.[14]

Concerning the age groups, the range of patients age registered in this study was 35 to ≥75 years with a mean age (57.73 ± 13.05 years) and most subjects that have the highest number of isolates were aged (55–64) years 33(27.7%), whereas subjects aged ≥75 years has the least

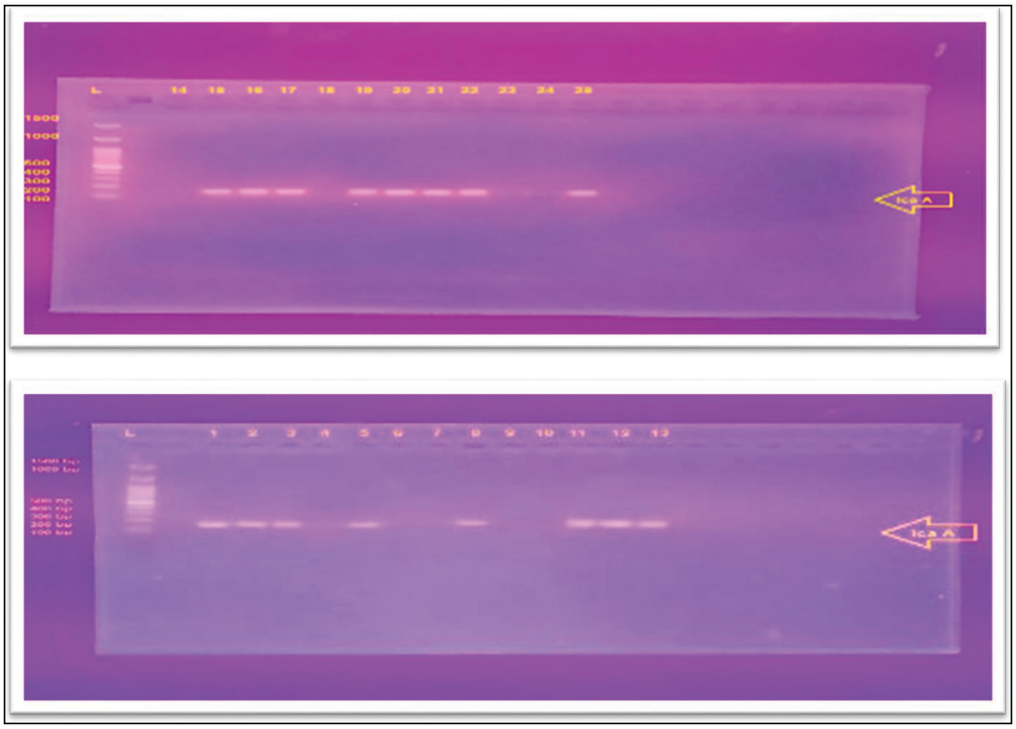

Figure 3: Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *ica*A gene (151 bp) using 1.5% agarose gel, 70 V for 50 min

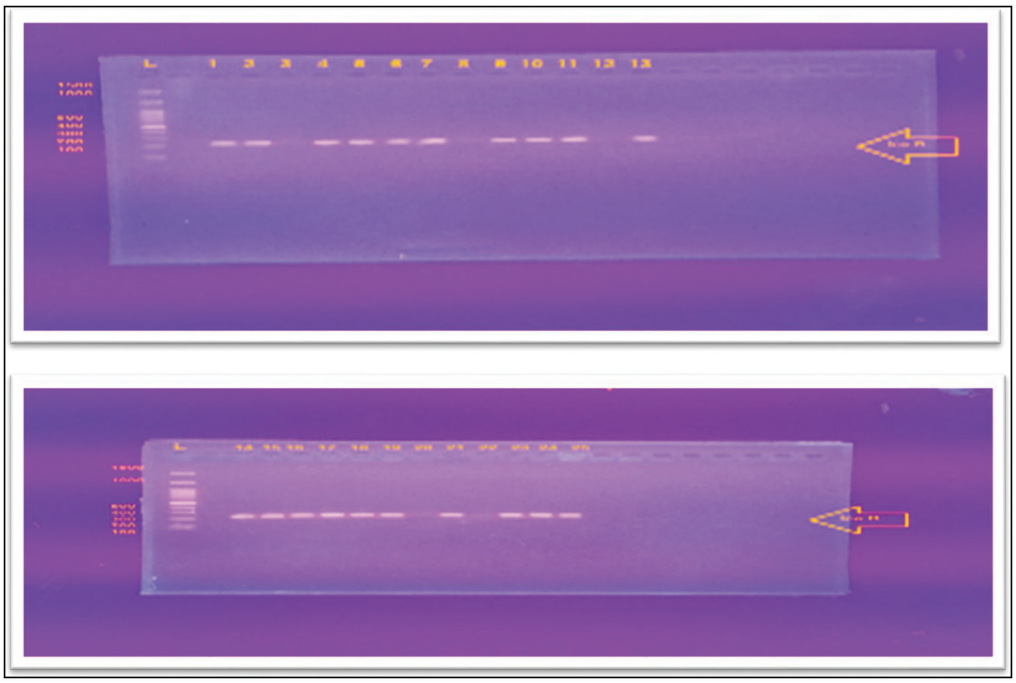

Figure 4: Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *ica*D gene (211 bp) using 1.5% agarose gel, 70 V for 50 min

number of isolates 18 (15%). This distribution of isolates in the current study is approach to Kadhim,[15] in a local study who reported that all of the DFU patients were older than 41 years.

The polymicrobial infection rates in the current study reached 82%, whereas monomicrobial rates were restricted to 18%. Our findings are higher than those reported by Ogba *et al.*[6] where polymicrobial infections reach 72% in

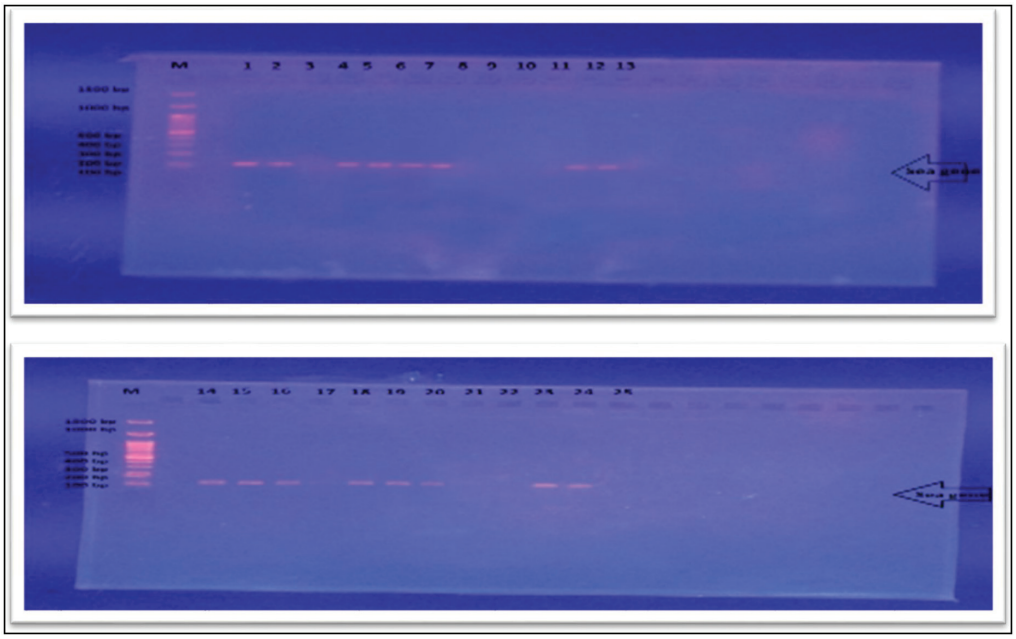

Figure 5: Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *sea* (102 bp) using 1.5% agarose gel, 70 V for 50 min

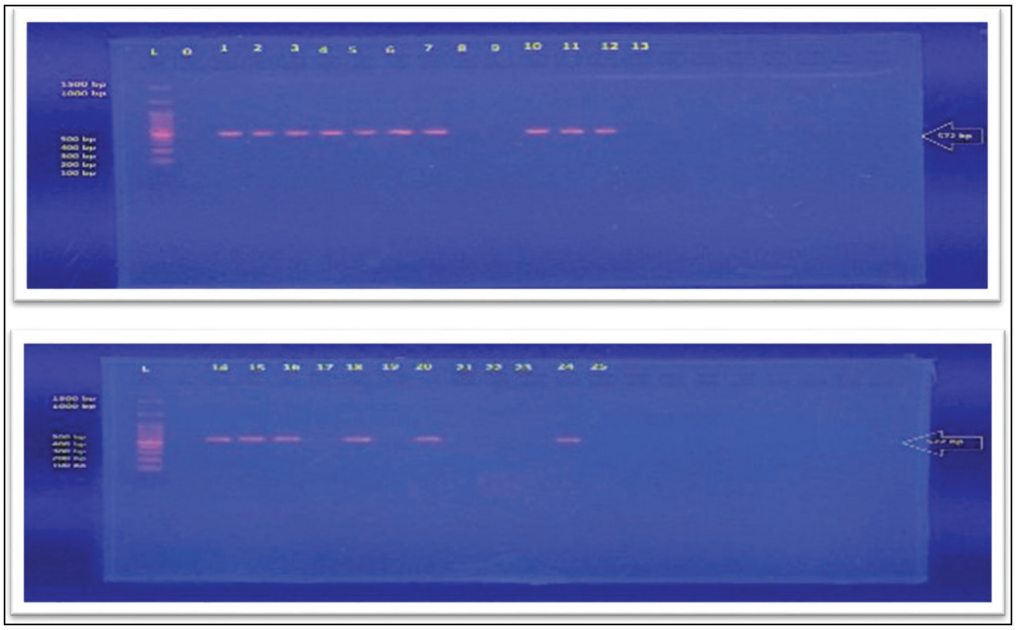

Figure 6: Electrophoresis of the PCR reaction product of *S. aureus* using the specific primer of the *erm*C gene (572bp) using 1.5% agarose gel, 70 V for 50 min

Nigeria. Globally, the number of isolates found in DFI varies widely. Polymicrobial infection rates are more prevalent in a study done in Mumbai, India.[16]

The biochemical and microscopical tests were applied to identify the exact bacterial species obtained from DFI and the isolation procedure resulted in the predominance of *S. aureus* 25 (21%) isolates. These results agreed with Ogba *et al.*[6] who obtained *S. aureus*, *P. aeruginosa*, *P. mirabilis*, *E. coli*, and *K. pneumoniae* in a percentage of 31.9%, 24.7%, 17.5%, 13.4%, and 12.4%, respectively, in a study performed in Nigeria. This variation in bacterial profiles isolated from patients with DFUs could be attributed to variations in sample collection method, geographical region, treatment therapy, and severity of infection.[14]

In the current study, 60% of *S. aureus* isolates were resistant to penicillin, piperacillin, amoxicillin–clavulanic acid, ceftazidime, cefepime, cefotaxime, ceftriaxone, imipenem, meropenem, and aztreonam that belong to β-lactam

antibiotics. All of these isolates were MRSA. These results agreed with Dwedar *et al.*[17] in Egypt, who reported that all MRAS strains isolated from DFI were 100% resistant to antistaphylococcal β-lactams, combined penicillins, and third-generation cephalosporins and cabapenim. *S. aureus* can resist β-lactams by several methods, such as the synthesis of β-lactamases, which hydrolyzes the β-lactam ring of penicillin, thereby executing it inactive.[18] In our study, 84% of *S. aureus* isolates were found to be sensitive to levofloxacin, whereas 73.34% of *S. aureus* were sensitive to levofloxacin according to Shareef *et al.*[19] in India. Levofloxacin belongs to fluoroquinolones, which are classified as bactericidal drugs. They inhibit the activity of topoisomerase II (gyrase) and topoisomerase IV enzymes, which are responsible for DNA super-coiling and recapitalization.[20] In the current study, 64% of *S. aureus* isolates were resistant to erythromycin, whereas Neama *et al.*[21] in a local study reported that 70.7% of *S .aureus* were resistant to erythromycin and 59% of *S. aureus* were resistant toward erythromycin in a local study done by Hamza and Fazaa[22] in 2023.Erythromycin belongs to macrolides group and the resistance of bacteria to this antibiotic can be illustrated by two mechanisms: first, by producing an esterase enzyme that disrupts the lactone ring of the antibiotic or makes an alteration in antibiotic structure by transferring the functional group such as acyl, ribosyl, and phosphoryl through making an alteration in antibiotic structure by action of glycosyltransferase. Second, through the modification of the antibiotic target by methylation of 23SrRNA.[9] Concerning rifampin, 52% of *S. aureus* isolates were sensitive in this study and that was approached by Chai *et al.*[10] findings reported that 54.2% of *S. aureus* were sensitive to that antibiotic in China. Rifampin is a wide-spectrum antibiotic against bacterial pathogens and its mode of action is summarized by its ability to inhibit bacterial RNA-polymerase, which can directly block the pathway of ribonucleic acid (RNA) elongation.[11] In our study, 52% of *S. aureus* isolates were resistant to ciprofloxacin, whereas in a local study performed by Al-Charrakh and Al-Safaar,[12] it was found that *S. aureus* was 47.6% resistant toward ciprofloxacin.

According to the microtiter plate method, 60% of *S. aureus* obtained from DFI in the current study were strong producers of biofilm, whereas 40% of them were moderate producers. This result is not in agreement with a study performed by Mamdoh *et al.*,[23], it has been found that 59% of *Staphylococcus* isolates were strong producers, whereas 32.8% were moderate and 8.2% were weak producers in a study performed in Egypt. Mahmoudi *et al.*[24] in Iran reported that 58% of the *S. aureus* were strongly formers for biofilm, 22% were moderate producers, 14% were weak biofilm producers, and 6% were nonproducers.

In this study, the PCR technique was used to detect the presence of the *mec*A gene in *S. aureus* and the presence of this gene indicates that *S. aureus* is methicillin-resistant. Figure 2 shows the electrophoresis of the PCR products, through which it is clear that the primer of the *mec*A gene was successful in amplifying this gene through the appearance of a PCR product of 310 bp in size. This result agreed with Elhassan *et al.*[25] who could obtain the same result and success in the electrophoresis of the *mec*A gene of *S. aureus* obtained from different clinical sources in a study performed in Saudi Arabia. Upon examining Figure 2 it is clear that 15 isolates of the bacteria under study contain a gene *mec*A, and comparison of these isolates with susceptibility tests has found that the mentioned isolates were resistant toward oxacillin and β-lactam antibiotics. The methicillin resistance in *S. aureus* is linked with the presence of the *mec*A gene encoding penicillin-binding protein (PBP2a) in mobile SCCmec.[26] The expression of *mec*A and PBP2a is a good sign for guessing oxacillin resistance in *S. aureus*.[27]

This study also included the use of PCR technique to detect the presence of the *ica*A gene, which is responsible for biofilm formation. Figure 3 shows that *ica*A primer was successful in amplifying this gene through the appearance of a PCR product of 151 bp in size in 64% of *S. aureus*. This result agreed with Raheema *et al.*[28] in a local study that obtained the *ica*A gene of the same product size. Regarding the strength of biofilm formation and the presence of the *ica*A gene, our findings revealed that 9/15 isolates (60%) of the strong biofilm producers contained the *ica*A gene, whereas 7/10 isolates (70%) of the moderate biofilm formers possessed *ica*A gene. This is similar to Abdel-Shafi *et al.*[29] findings, which documented that 47.37% of the strong *S. aureus* biofilm producers possessed the *ica*A gene.[28] The *ica*A gene is identified to encode *N* acetyl-glucosamine transferase transmembrane synthesizing Poly N-acetylglucisamine polymers.[29] The *ica*A gene regulates the formation of exopolysaccharides (EPS) in biofilms. This EPS imposes the adhesion of the bacteria and can assist as a shelter against the host immune system and antibiotics treatment.[30]

By using the PCR technique also, the presence of the *ica*D gene was detected, which is responsible for biofilm formation. Figure 4 shows the *ica*D gene was successfully amplified, which has been indicated by the presence of a PCR product size of 211 bp in 80% of *S. aureus* isolates. This result is in agreement with Mahmoudi *et al.*[24] who could have obtained the same PCR product size through their electrophoresis of the *ica*D gene in their study in Iran. Concerning the strength of biofilm production with the presence of the *ica*D gene, our results reflect that among the strong biofilm producers, 12/15 (80%) of the isolates contained the *ica*D gene, whereas 8/10 (80%) of the moderate biofilm producers contained with *ica*D gene. Our result agrees with Mamdoh *et al.* (2023) in Egypt who reported that 79.4% of strong biofilm producers contained the *ica*D gene and 75% of intermediate biofilm producers

contained the *ica*D gene,[23] whereas Haddad *et al.*[30] documented that the highest rate 60.9% of moderate biofilm producers contained the *ica*D gene and 65% of strong biofilm producers possessed the *ica*D gene in their study performed in Tunisia

In this study, the presence of the *sea* gene, which is responsible for the production of enterotoxin is detected by using the PCR technique. Figure 5 displays the successful amplification that has been distinguished by the appearance of bands related to *sea* gene PCR product size of 102 bp in 64% of *S. aureus* isolates. *sea* might have a chief role in atopic dermatitis by prompting the upregulation of adhesion molecules and provoking inflammatory responses in endothelial cells and keratinocytes.[31]

Finally, the presence of the *erm*C gene was detected by using of PCR technique. Figure 6 shows the successful amplification of the *erm*C gene product size of 572 bp in 64% of *S. aureus* isolates obtained from DFI in the current study. This was in agreement with Gushiken *et al.*[32] in Brazil reported that 64.29% of *S. aureus* in their study contained the *erm*C gene.Modification of the ribosomal target site leads to a broad-spectrum resistance to macrolides, whereas efflux and enzymatic inactivation are of less importance. However, macrolides are considered by a resistance mechanism showing different phenotypic expression, which is essential in their interpretation.[33]

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Nil.

Conflicts of interest
There are no conflicts of interest.

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