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ORIGINAL ARTICLE

Antibiotic susceptibility and biofilm forming ability of *Staphylococcus aureus* isolated from Jordanian patients with diabetic foot ulcer

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ABSTRACT

Background and Objectives: Microbial biofilm is characterized by the irreversible attachment of planktonic cells to a surface and is usually associated with high antimicrobial resistance with worsening the wound healing. The objective of the study was to determine the prevalence of *Staphylococcus aureus* in diabetic foot ulcers (DFUs) of diabetic patients and to investigate antibiotic susceptibility patterns of these isolates. In addition to screen biofilm forming ability of isolated *S. aureus.*
Materials and Methods: A total of 112 non-healing wound swabs of diabetic foot patients were collected and cultured on different culture media to identify and characterize 98 isolates. The *S. aureus* isolates were examined for their antibiotic susceptibility to different antimicrobial agents. Furthermore, *S. aureus* isolates were evaluated for their biofilm production capability using the Tissue Culture Plate Method (TPC). The level of *icaA* gene expression was determined by RT-PCR.
Results: The results of this study showed that these non-healing wounds yield positive cultures, with an average of 1.67 organisms per sample. The isolates showed highest resistance against oxacillin (95.2%) and lowest resistance against linezolid (3.7%). All isolates were biofilm producers and a significant association with the *icaA* gene expression level was recorded.
Conclusion: This study showed that *S. aureus* isolates have a great ability to produce biofilms that are associated with the chronicity of wounds in diabetic patients. Routine screening for biofilm formers in chronic wounds and their antibiotic susceptibility testing will help in early treatment and prevent any other complications.

Keywords: Antimicrobial resistance; Biofilm; Diabetes; Diabetic foot; *Staphylococcus aureus*

INTRODUCTION

Neuropathy and vascular problems are common complications of diabetes mellitus (DM), which may result in diabetic foot infections (1). Unhealed chronic wounds like diabetic foot ulcers, pressure ulcers, and venous leg ulcers, are major worldwide healthcare problems (2, 3). DM is a systemic metabolic disease displaying significant incidence worldwide, and is characterized by increased blood glucose levels (4).

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Diabetic foot complications have the largest clinical influence of all other medical complications associated with diabetes; 85% of limb amputations are preceded by a nonhealing ulcer (5). Studies estimated that approximately 25% of patients will develop one or more foot ulcers (DFUs) during their lifetime (6, 7). Once the skin protective layer is broken, deep soft tissues will be exposed to bacterial infections due to the bacterial colonization with the involvement of joints or bones; as a result, osteomyelitis will occur (8, 9). Mortality and morbidity are remarkably affected by DFUs (10), and patients' quality of life is negatively influenced leading to reduced physical, physiological, and social functions (11). In DFUs, the most frequently isolated microorganisms may include *Staphylococcus, Streptococcus, Enterococci, Enterobacteriaceae,* and *Pseudomonas aeruginosa* species (12, 13).

Recent studies have shown that one of the most prevalent colonizers among DFUs is *S. aureus* (14, 15). *S. aureus* uses virulence factors (VFs) for defense and escape from the immune system. Generally, biofilm production by *S. aureus* is considered as it's major defense mechanism leading to wound chronicity with suppressed wound healing (16). Therefore, better management methods and therapy could be developed if the bacterial biofilms and mechanism to eradicate them are understood (17). Naturally, the structure of biofilm is genetically resistant to antimicrobial agents and hard to remove from the infected tissue, since they can be up to ten thousand times less susceptible to antimicrobial agents than free-floating bacteria (18). Poly-intercellular adhesion (PIA) molecules are encoded by a locus called *ica*, which includes *ica*ABCD genes, among the *ica* genes *icaA* and *icaD* are known to be responsible for biofilm formation in *S. epidermidis* and *S. aureus* (19). To manage such infections by using proper antibiotics, appropriate screening of multi-drug resistant organisms (MDROs), their antimicrobial susceptibility patterns and detection of biofilm formation are required (20). DFUs and DFIs are primarily multidrug-resistant, poly-microbial infections caused by the strains of higher ability to form biofilms as a significant virulence factor and hence often leads to treatment failure (21). To our knowledge, few studies in Jordan have demonstrated *S. aureus* biofilm production and its co-relationship with antimicrobial resistance pattern (22, 23). However, less information is available on corelating level of biofilm genes expression with drug resistance. Therefore, considering this, the present study aimed to investigate incidence of bacterial species especially *S. aureus* in chronic pus samples of diabetic foot ulcer patients and to establish their occurrence in relation to gender and age. Also, to determine drug susceptibility profile to find out effective antimicrobial drug against muti-drug resistant and strong biofilm forming isolates. We further attempted to correlate multi-drug resistance and biofilm formation with the level of *icaA* gene expression.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 112 pus samples were collected from patients with unhealed diabetic foot ulcers using sterile cotton swabs, after official approval for samples collection was obtained from the National Centre for Diabetes Endocrinology and Genetics. This study was approved by the Institutional Review Board committee (IRB) at Al-Balqa Applied University. The pus samples were put into a transportation medium to be cultured later. Out of these samples, 98 bacterial isolates were obtained. Using a sterile loop, the pus samples were streaked on different kinds of media (Chocolate agar, MacConkey, Mannitol salt agar, and blood agar), then incubated for 24-48 hours at 37°C. After the incubation period, the isolates were discriminated from each other on the basis of morphological appearance and characteristics such as consistency, size, colony color, texture. The isolates were identified based on Gram staining, morphological and biochemical characterization according to Bergey's manual (24).

Antimicrobial susceptibility testing. As shown in Table 1, fifteen antimicrobial agents purchased from Bioanalyse (Ankara, Turkey), were used for susceptibility testing of all *S. aureus* isolates. Disc diffusion assays as recommended by Clinical and Laboratory Standards Institute (CLSI) documents M02-A12 was used with some modifications to assess the sensitivity of the bacterial strains. Briefly, 100 µL of 0.5 McFarland cell suspension was spread onto NA plates. Next, antibiotic drug discs were placed over the agar surface and incubated at 37°C for 24 h. Each experiment was conducted in triplicate and the average diameter of the zone of inhibition around the discs was calculated in mm. The isolates were classified into resistant, intermediate, and sensitive by following the guidelines of CLSI.

Table 1. Antibiotic discs used for antibiotic susceptibility testing for *S. aureus* isolates.

Antibiotic	Symbol	Concentration
Norfloxacin	NOR	10 µg
Oxacillin	OX	1 µg
Tigecycline	TGC	15 µg
Teicoplanin	TEC	30 µg
Erythromycin	E	15 µg
Amikacin	AK	30 µg
Linezolid	LZ	30 µg
Gentamycin	GM	10 µg
Clindamycin	DA	2 µg
Rifampin	RF	5 µg
Levofloxacin	LEV	5 µg
Chloramphenicol	CL	30 µg
Ciprofloxacin	CIP	5 µg
Ampicillin	AM	10 µg
Cefazolin	CZ	30 µg

Tissue culture plate method (TCP). TCP as adapted by Hassan et. al. (25) is considered as the most widely used and a standard method for the detection of biofilm formation. In the present study, all the isolates were tested in triplicate using this screening test to evaluate their ability to produce biofilms. A broth without bacterial inoculum served as negative control whereas a standard reference strain *S. aureus* ATCC25923 (a known biofilms former strain) is used as positive control. OD values represent an indicator of bacteria adhering to the surface and biofilm formation. OD ≤ 0.617 was considered a non-biofilm producer. Whereas 0.617< OD ≤ 1.234 was considered a moderate biofilm producer and OD > 2.4 as strong biofilm producer.

Real-time PCR assay: isolates preparation and RNA extraction from *Staphylococcus aureus*. The RNA extraction was obtained by using SV total RNA isolation system (Promega, UK) by following the manufacturer's procedure. Briefly, overnight grown culture, at 37°C, on nutrient agar of 28 isolates with different degrees of ability to form biofilms. Fresh bacterial isolates were inoculated in tubes containing 5 ml of sterile TSB supplemented with 1% glucose, and then it was adjusted to 0.5 McFarland, and incubated for 18 hours at 37°C. Using sterile 96-well tissue culture plates, a volume of 1000 µl of the broth was dispensed into the wells, and then the plates were covered by lids and incubated for 24 hours at 37°C. After incubation, the plates were washed twice with PBS (PH= 7.2), left to dry, then fixed with ethanol for 15 minutes and further processed to obtain RNA extracts as adapted by the authors (18, 26, 27). The elution tubes containing purified RNA were stored at -70°C.

Assessment of isolated RNA quality and quantity. NanoDrop (Thermo Fisher Scientific, USA) was used to quantify the concentration and purity of isolated RNA to evaluate the integrity of samples for further analysis in RT-qPCR. RNA concentration and RNA purity levels were detected at 260/280 nm absorbance ratio. A_{260}/A_{280} ratio of approximately 2.0 was suggestive that the RNA sample is pure and accepted for conversion to cDNA.

Complementary DNA (cDNA) synthesis. RNA samples were converted to cDNA following the manufactures instructions of QuantiTect Reverse Transcription Kit (Qiagen, Germany). Genomic DNA elimination reaction was prepared by adding 2 µl of gDNA Wipeout Buffer, 4 µl of templet RNA, and RNase-free water, and incubated for 2 minutes at 42°C, then placed directly on ice. The reverse-transcription master mix was prepared by adding 1 µl RNase inhibitor, 2 µl of $MgCl_2$, 2 µl dNTPs, RT Primer mix, and 14 µl genomic DNA elimination reaction. Then, 14 µl RNA templets were added to each tube containing a reverse-transcription master mix and incubated for 15 minutes at 42°C, and 3 minutes at 95°C using a thermal cycler (Thermo Fisher Scientific, USA), to inactivate RTase. Samples containing cDNA were preserved at -20°C to be used later (28, 29).

Gene expression profiling by RT-qPCR. The *recA* reference gene was used as an internal control to calculate the ΔCT value. Primers of target and housekeeping gene that were used in the study are shown in Table 2. RT-qPCR master mix was prepared for each reaction by combining the components taking into consideration the thermal cycling conditions are shown in Tables 2 and 3.

Statistical analysis. Experiments were repeated three times, and the data were analyzed using Statistical Package for Social Sciences (SPSS). Version 22.0 (IBM Corporation, Armonk, NY). Frequencies and percentages were used for categorical variables. Pearson's correlation test was utilized for the

Table 2. Primers of target gene and housekeeping gene used in the study.

Gene name	Amplicon size (bp)	Annealing temperature (C°)	Primer sequence (5'-3')
icaA	188	59°	F: ACACTTGCTGGCGCAGTCAA R: TCTGGAACCAACATCCAACA (DuKanovic et al. 2022)
recA (housekeeping gene)	229	55°	F: AAGTACGTCGTGCAGA R: TGACCCATTCGTTCGC (30)

Table 3. The program of quantitative PCR for housekeeping gene and *icaA* gene.

Step	Temperature	Time	Number of cycles
Initial denaturation	95°C	5 minutes	1
Denaturation	95°C	15 seconds	40
Annealing	60°C	1 minute	40
Extension	72°C	20 seconds	40

association between the continuous variables. Moreover, for the demographics one-way ANOVA test was used.

RESULTS

Gender and age distribution. A total of 98 clinical isolates of both Gram-positive and Gram-negative bacteria were obtained from ulcers of diabetic patients including both males and females (n= 58) and (n= 40). The male group was the dominant in the study with a percentage of 59.2%, compared with females 40.8%. Out of 98 patients who were enrolled in this study, 13 (13.3%) were 50 years old or less, 19 (19.4%) were between 51 and 60 years old, and 66 (67.3%) were >60 years old.

Bacterial isolates characteristics. Among the ninety-eight isolates, both poly-microbial and mono-microbial growths were observed in the diabetic foot ulcers of the patients. Mono-microbial growth was 57 (58.2%) and was more than poly-microbial growth of 41 (41.8%). Gram-positive isolates were more prevalent than Gram-negative isolates. Out of total isolates, Gram-negative isolates were 39 (39.8%) whereas Gram-positive isolates were 59 (60.2%) with *S. aureus* being the most prevalent among the Gram-positive isolates 32 (32.7%).

Antimicrobial resistance pattern of isolated *S. aureus*. Antimicrobial susceptibility testing was performed for 32 different *S. aureus* isolates to determine their antimicrobial profile against 15 antimicrobials. *S. aureus* isolates showed high rates of resistance to oxacillin (95.2%), clindamycin (68.7%), erythromycin (65.6%), cefazolin (60.2%), levofloxacin (50.2%), ciprofloxacin (48.1%), gentamycin (46.9%), and ampicillin (45.3%). Whereas antibiotics that are effective and sensitive were linezolid (3.7%), rifampin (5.1%), chloramphenicol (6.7%), norfloxacin (14.1%), teicoplanin (15.6%), amikacin (18.8%), tigecycline (31.2%) (Fig. 1).

Assessment of biofilm production. Regarding biofilm production assays, the TCP method was performed and identified 10 (31.25%) isolates as strong biofilm-producer isolates, 14 (43.75%) as moderate biofilm-producer isolates, and 8 (25%) as non-biofilm producer isolates (Fig. 2). Out of 32 isolates of *S. aureus*, 24 (75%) were biofilm producers and 23 (71.9%) were multi-drug resistant. The bivariate analysis showed a significant statically correlation between the capability of the isolate to produce biofilm and their multi-drug resistance (p= 0.015).

RT-PCR results. In this study, the level of *icaA* gene expression was evaluated using the RT-PCR technique, in which the *icaA* gene is considered as a major gene that is responsible for polysaccharide production in *S. aureus*. The expression levels were investigated in 28 isolates selected carefully based on the degree of biofilm production; 7 isolates each from strong, moderate, weak, and non-biofilm producers. The fold change in expression of our target gene among the isolates was calculated using the Livak method ($2^{-\Delta\Delta CT}$) by calculating the ΔCT which is the difference between the CT value of the gene of interest and the CT value of the reference (housekeeping) gene. These results showed that CT values of the *recA* housekeep-

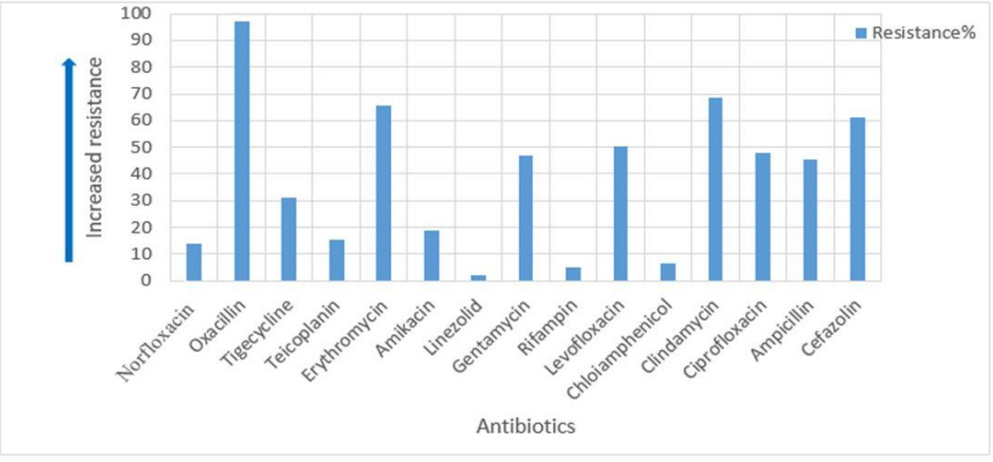

Fig. 1. Antimicrobial resistance pattern of isolated *S. aureus.*

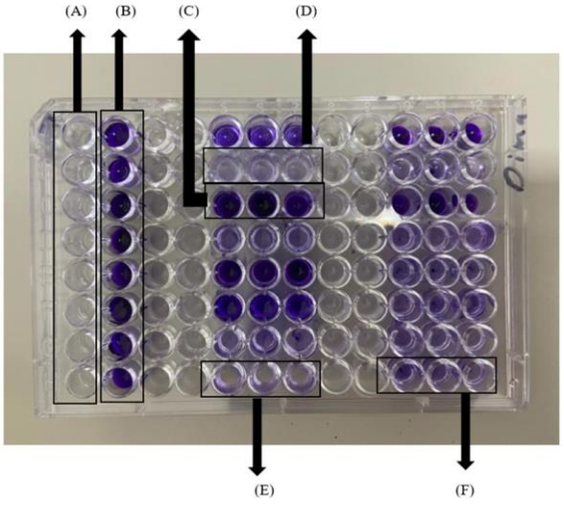

Fig. 2. 96-well plate for TCP method stained with crystal violet. (A): Negative control (B) Positive control (C) Strong biofilm producer (D) weak biofilm producer (E) Non-biofilm producer (F) Moderate biofilm producer.

ing gene were obtained in all of the *S. aureus* isolates. CT values of the *recA* gene ranged between 16.09 and 18.99 with an average of 17.4. In comparison, CT values results of the *icaA* gene in the strong biofilm producer's strains ranged between 22.97 and 25.94 with an average of 24.49 by a highly significant fold 4.59-7.4 ($P<0.01$) when compared with *recA*. While, in moderate biofilm producer's strains, CT values results ranged between 25.93 and 27.17 with an average of 26.66 by a 1.58- 3.38 fold. Whereas in weak biofilm producer strains, CT values ranged between 28.21 and 29.25 with an average of 28.59 by 1.14-1.46 fold as shown in the (Tables 4-6).

DISCUSSION

DFUs are most frequent complications among diabetics worldwide, which is considered as the main reason for hospitalization among diabetic patients, with a huge financial burden (31). Bacterial adhesion in chronic wounds is known to be a significant virulence factor contributing to chronic infections. Further, the capability of the pathogens to form biofilms helps them to escape the immune system and is considered as the primary leading cause of chronic infections (21). In fact, the severity of infection is determined by host-related disorders and pathogen-related factors such as microbial load, resistance of the pathogens to antimicrobial agents, and virulence factors used by the pathogen. Considering this we carried out this study to obtain the microbiological profile of 112 wound samples from DFUs patients. Our study highlighted that most of the diabetic people who developed ulcers were elderly people above 60 years. Similar observation was reported by Ponirakis et al. (32). It is claimed that elderly diabetic patients develop progressive diseases, poor vision. Further, aging may result in peripheral circulation disorders, and thereby resulting in gait abnormalities including lower extremity sensory abnormalities, making them a high-risk population for diabetic foot ulcers (33).

We observed that males were more prone to develop foot infections than females with a percentage of 59.2% and 40.8%, respectively. Similarly, the findings by Kim and Han (34), reported that out of 131 diabetic patients, 91 were males and 40 were females. Research has recently shown that there is link

Table 4. Fold changes in expression of the target gene among the strong biofilm producers isolates.

Isolates	After (strong group)			Before			ΔΔCt	$2^{-\Delta\Delta CT}$
	TG (gene)	HK	ΔCt	TG gene	HK	ΔCt		
S1	23.87	16.56	7.31	29.014	19.265	9.749	-2.439	5.42265731
S2	24.96	17.41	7.55	29.014	19.265	9.749	-2.199	4.59160966
S3	25.94	18.99	6.95	29.014	19.265	9.749	-2.799	6.95957882
S4	23.91	16.91	7	29.014	19.265	9.749	-2.749	6.72251002
S5	23.96	16.44	7.52	29.014	19.265	9.749	-2.229	4.68808913
S6	22.97	16.11	6.86	29.014	19.265	9.749	-2.889	7.40756818
S7	25.83	18.39	7.44	29.014	19.265	9.749	-2.309	4.95539479

Table 5. Fold changes in expression of the target gene among the moderate biofilm producing isolates.

Isolates	After (Moderate group)			Before (control group)			ΔΔCt	$2^{-\Delta\Delta CT}$
	TG (A)	HK	ΔCt	TG (A)	HK	ΔCt		
M1	25.99	16.97	9.02	29.014	19.265	9.749	-0.729	1.65748981
M2	27.17	18.91	8.26	29.014	19.265	9.749	-1.489	2.80694345
M3	26.91	18.56	8.35	29.014	19.265	9.749	-1.399	2.63718723
M4	27.18	18.87	8.31	29.014	19.265	9.749	-1.439	2.71132865
M5	26.49	18.23	8.26	29.014	19.265	9.749	-1.489	2.80694345
M6	26.96	18.97	7.99	29.014	19.265	9.749	-1.759	3.38463439
M7	25.93	16.85	08	29.014	19.265	9.749	-0.669	1.5899705

Table 6. Fold changes in expression of the target genes among the moderate biofilm producers.

Isolates	After (weak group)			Before (control group)			ΔΔCt	Fold Change
	TG (A)	HK	ΔCt	TG (A)	HK	ΔCt		
W1	28.27	18.95	9.32	29.014	19.265	9.749	-0.429	1.34630007
W2	28.42	18.87	9.55	29.014	19.265	9.749	-0.199	1.14790241
W3	28.21	18.97	9.24	29.014	19.265	9.749	-0.509	1.42306346
W4	28.49	19.09	9.4	29.014	19.265	9.749	-0.349	1.27367748
W5	29.18	19.98	9.2	29.014	19.265	9.749	-0.549	1.46307122
W6	29.25	19.86	9.39	29.014	19.265	9.749	-0.359	1.2825366
W7	28.32	18.88	9.44	29.014	19.265	9.749	-0.309	1.2388487

between testosterone hormone and development of type 2 diabetes in men. Type 2 diabetes has a direct correlation with increased risk of visceral fat deposition due to low testosterone levels in men, leading to increased type 2 diabetes. However, in women this hormone is extremely low and has no effect on metabolism especially after menopause age in elderly women (35, 36).

In this study, we observed that wound samples were dominated by both mono-microbial and poly-microbial infections, and a total of 98 Gram-positive and Gram-negative bacterial isolates were recovered. Out of 98 samples, Gram-positive bacteria accounted for 60.2%, with *S. aureus* being the most frequent isolate among the Gram-positive isolates, many previous studies have reported similar findings who reported that *S. aureus* was the most frequent isolated pathogen among the Gram-positive (37). Moreover, Costa et al. (38) showed that the most frequently isolated bacteria among Gram-negative isolates was *P. aeruginosa*. Whereas contrary to our findings, Jain et al. (39) found that Gram-negative species were more frequent than Gram-positive with the incidence of 51.2% and 32.3%, respectively, and *Enterobacte-*

riaceae being the most common isolated pathogen. These differences in the results of wound bacteriology and types of dominant species may be related to geographic differences, variations in the environment, socioeconomic differences, or previous antibiotic therapy (40).

Furthermore, the antibiotic susceptibility profiling suggested that most preferred antibiotics against *S. aureus* could be in the order of linezolid >chloramphenicol >rifampin >teicoplanin >amikacin >norfloxacin >tigecycline. Thus, linezolid could be proposed as a highly effective antimicrobial agent to treat wound infections for diabetic patients who are infected with *S. aureus.* This observation finds support from a study conducted in Nepal by Belbase et al. (41) showing higher sensitivity of *S. aureus* towards linezolid. However, *S. aureus* isolates in our study were resistant to antibiotics in the order of oxacillin >clindamycin >erythromycin >cefazolin >levofloxacin >ciprofloxacin >gentamycin >ampicillin. Jouhar et al. (42) investigated the microbiological profiles and antibiotic susceptibility pattern for 179 DFIs and found a high resistance rates for oxacillin. In contrast, results by Palomo et al. (43) revealed that *S. aureus* in DFI was susceptible to oxacillin and ampicillin. These variations and differences in antibiotic susceptibility profiles may be related to the variation in sample size, sample population, hospital care protocols, infection control activities, and the educational level of glycemic control, especially in developed countries.

Considering that infections caused by biofilm-producer strains are difficult to be healed because they show higher resistance towards antimicrobials, which is significantly associated with the chronicity of the wound. We screened our *S. aureus* isolates on the biofilm formation capability, and the profiling of responsible genes. Moreover, the majority of the bacterial species were MDR. Regarding biofilm formation assessment among the isolates, the vast majority of the isolates were a biofilm producer, which is a major virulence factor that induces chronic infections of *S. aureus* and prevents antimicrobial agents from penetrating cells, consequently, contributing to the high antimicrobial resistance. Finally, there was a significant association between MDR in biofilm-producing strains. We found that significant number of *S. aureus* isolates produced biofilm as in agreement with the results of Bose et al. (44). In our study, high incidence (71.9%) of MDROs could be associated with non-healing chronic wounds and ulcers in diabetic patients as also suggested by Murali et al. (45). Moreover, our findings demonstrated the correlation between MDROs and biofilm-producer strains, and there was a high and significant association with biofilm-producer strains compared with non-biofilm producers (P= 0.015). The other reports also support our observation (46, 47). Regarding gene expression results, remarkably, there was a relation between *icaA* gene expression level and the ability of forming biofilms. The strong biofilm producer strains had a higher level of gene expression when compared with moderate and weak strains. A study conducted by Mohammed and Radif (48), on 57 wound samples, showed that the level of gene expression in the strong biofilm-producing strains, was significantly higher (6.50) when compared with the weak and moderate isolates (1.23) and (1.62), respectively. (49) studied the influence of various biomaterials on staphylococcal adhesion and how *icaA* gene expression in *S. aureus* isolates has been affected. They observed that after 3 to 6 hours the expression level of the *icaA* gene increased and found that strain with weak biofilm formation ability display a low level of *icaA* expression and could be considered as a planktonic. Therefore, it is suggested that screening for biofilm formation should be a routine procedure in chronic non-healing wounds to choose the suitable and appropriate treatment.

CONCLUSION

DFUs are serious life-threatening complications of diabetes that is associated with a reduction of health-related quality of life and contributes to the high rate of mortality and morbidity among diabetic patients. Considering this our studies has highlighted that non healing wound infections are more frequent in elderly male diabetic patients. Such infections are predominated by the Gram- positive MDROs especially oxacillin resistant *S. aureus* isolates. This multi drug resistance could be the result of strong biofilm forming ability of isolates exhibiting increased expression of *icaA* genes. It is considered as a major virulence factor that induces chronic infections of *S. aureus* and prevents antimicrobial agents from penetrating cells, consequently, contributing to the high antimicrobial resistance. We conclude that such kinds of infections could be better treated by using antibi-

otics like linezolid, chloramphenicol, and rifampin. Overall, the findings of the current study are expected to assist health workers to manage such infections by offering appropriate antibiotic therapy, which will reduce time, effort, and cost of the treatment.

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