Infection and Drug Resistance

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ORIGINAL RESEARCH

Clinical and Bacteriological Analyses of Biofilm-Forming Staphylococci Isolated from Diabetic Foot Ulcers

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Background: Diabetes mellitus is a chronic disease that is associated with increased morbidity and mortality. Unfortunately, foot ulcers and amputations due to diabetes are very common in developing countries. The purpose of this study was to characterize the clinical presentation of diabetic foot ulcer (DFU) infections, isolate the causative agent, and analyze the biofilm formation and distribution of biofilm-related genes among isolated Staphylococci.

Material and Methods: The study included 100 diabetic patients suffering from DFUs attending Assiut University Hospital. Swabs were collected and antimicrobial susceptibility testing of the isolates was performed. Biofilm formation was tested phenotypically among staphylococcal isolates and the frequency of different biofilm genes was analyzed by PCR. Clinical presentations of diabetic foot ulcers were correlated with bacterial genetic characteristics. Spa types were determined using DNA Gear-a software.

Results: Microbiological analysis showed that 94/100 of the DFUs were positive for bacterial growth. The majority of infections were polymicrobial (54%, n=54/100). Staphylococci were the most commonly detected organisms, of which *S. aureus represented* 37.5% (n=24/64), *S. haemolyticus* 23.4% (n=15/64), S. *epidermidis* 34.3% (n=22/64) and other CNS 4.7% (n=3/64). Interestingly, co-infection with more than one species of Staphylococci was observed in 17.1% (n=11/64) of samples. A high level of antibiotic resistance was observed, where 78.1% (n=50/64) of *Staphylococci spp* were multidrug-resistant (MDR). Phenotypic detection showed that all isolated Staphylococci were biofilm-formers with different grades. Analysis of biofilm-forming genes among Staphylococci showed that the most predominant genes were icaD, *spa*, and *bap*. Isolates with a higher number of biofilm-related genes were associated with strong biofilm formation. Sequencing of the spa gene in *S. aureus* showed that our isolates represent a collection of 17 different spa types.

Conclusion: The majority of DFUs in our hospital are polymicrobial. Staphylococci other than *S.aureus* are major contributors to infected DFUs. MDR and biofilm formation are marked among isolates, which is paralleled by the presence of different categories of virulence-related genes. All severely infected wounds were associated with either strong or intermediate biofilm formers. The severity of DFU is directly related to the number of biofilm genes.

Keywords: diabetes mellitus, biofilm formation, Staphylococci, diabetic foot ulcers, *ica* genes

Introduction

Morbidity and mortality related to diabetes and its complications are rapidly emerging, particularly in developing countries. It is predicted that by 2040, there will be over 642 million people with diabetes in the world. Generally, diabetes is more prevalent in countries of low income.[1] Egypt is ranked the eighth country worldwide in the number of diabetic patients, and by 2045 it is expected to be the 6th country.[2] Unfortunately, foot ulcers and amputations are very common in developing countries. Infection of foot ulcers appears in more than half of all cases which required hospitalization and 20% of lower extremity infections will lead to

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amputation.[3] Amputation in people with diabetes is greater than those in non-diabetic people.[4] Someone loses a leg due to the complications of diabetes every 20 seconds in the world and more than 50% of these people will die within 5 years after such amputations.[5]

According to the International Diabetes Foundation, 40–60 million persons suffer from diabetic foot ulcers (DFUs), which markedly outnumbers the cases from 2015, that ranged from 9–26 million.[6] Several studies have shown that DFUs are classically polymicrobialy-infected, and *Staphylococcus aureus* (*S. aureus*) is the most frequently isolated pathogen.[7] These infected ulcers are characterized by dysregulated extracellular matrix remodeling, abnormal growth factors for angiogenesis, and excessive inflammatory reactions leading to impeding of the wound's recovery.[8] DFUs caused by antibiotic-resistant bacteria, particularly Methicillin Resistant Staphylococcus aureus (MRSA), are associated with more severe infections. The prevalence of MRSA in DFUs varies among countries, with an increasing rate in the developing countries.[9,10] Pathogen- and host-related factors (eg, immunity status, nerve damage, microbial virulence, and degree of antibiotic resistance) influence the prevalence, severity, and pathophysiology of the DFUs.[11]

A biofilm is a sessile layer generated from microbes and embedded in a matrix of extracellular polymeric substances and exhibits an altered phenotype with regard to growth, gene expression, and protein production.[12,13] The polysaccharide intercellular antigen (PIA) codes for biofilm-related genes in Staphylococci and was described as a specific polysaccharide antigen. Intercellular adhesion (*ica*) locus can control the production of PIA in vitro from UDP-N-acetylglucosamine.[14] Importantly, the *ica* locus, which includes the regulatory *icaR* and the biosynthetic genes *icaADBC,* is essential for the synthesis and virulence of the biofilms. However, biofilms can also occur in an *ica*-independent fashion and are upregulated in response to anaerobic growth which is provided by the biofilm environment.[15] In addition, Staphylococcal protein A (*spa*) production was reported to be essential for biofilm formation in an *ica*-deletion mutant of *S. aureus*. In these spa mutants, biofilm development could be recovered by the addition of an exogenous *spa*, indicating that spa does not need to be covalently anchored to the cell wall.[16] Furthermore, fibronectin-binding proteins (FnBPs) can mediate biofilm development via the major autolysin (*Atl*) and *sigB*. Additionally, S. aureus's biofilm-associated protein (Bap) and Bap-related proteins, which are distinguished by their high molecular weight, presence on the bacterial surface, and crucial virulence function, can promote biofilm development independently of PIA production through cell-to-cell aggregation.[17]

In *S. epidermidis*, PIA-independent biofilms were mediated through the accumulation-associated protein (*Aap*).[18,19] The *agr* quorum sensing system indirectly decreases initial biofilm formation by reducing the expression of genes encoding adhesion factors, which lead to less adherence. Also, *agr* tend to upregulate the expression of detergent-like peptides and nucleases that seem to increase biofilm detachment.[20] Induction of *agr* through an essential component of the quorum sensing system auto-inducing peptides (*AIP*) leads to the spread of seeding microorganisms in developed biofilms.[21]

The aim of this work was to isolate and identify bacterial pathogens associated with diabetic foot ulcers, to determine the antimicrobial susceptibility pattern of the isolated bacterial pathogens, to evaluate the ability of the isolated Staphylococcal species to form biofilms quantitatively, screen for genes that are responsible for biofilm formation and molecular typing of *S. aureus* strains to identify strain relatedness.

Materials and Methods

Ethical Consideration

Informed consent was obtained from all cases. This study complied with the Declaration of Helsinki and the study protocol was approved by the scientific ethics committee of the Faculty of Medicine, Assiut University (IRB no: 17101272).

Bacterial Isolates

This cross-sectional observational study was conducted over the duration of July 2018 to September 2019. A total of 100 diabetic patients attending Diabetes, Endocrine Centre and Vascular Surgery outpatient clinics in Assiut University Hospitals and suffering from DFU were enrolled. Sterile disposable cotton swabs were obtained from diabetic foot ulcers. Standard procedures were used for isolating, processing, and identifying all isolates.[22] *Staphylococcus aureus, Staphylococcus epidermidis, and Staphylococcus haemolyticus* identification were confirmed using a multiplex PCR method.[23]

Phenotypic Testing of Biofilm Formation

The microtiter plate (MTP) was used for the quantitative evaluation of biofilm formation. Briefly, a bacterial suspension was prepared in trypticase soy broth containing 1% glucose (Merck, Darmstadt, Germany). Culture was added to a 96-well plate and incubated overnight at 37°C. Then, supernatant was discarded, wells were washed with PBS and incubated at 65°C until dry. Adherent bacteria were stained with 0.1% crystal violet dye (Sigma Chemical Co., St Louis, MO, USA), then washed with deionized water to get rid of the excess dye. For solubilization, 70% ethanol with 10% isopropyl alcohol was added and the optical density was read at 570 nm by an ELISA plate reader (BioTek, Germany). Each test was performed in triplicate.[23]

Screening for Biofilm related Determinants

The presence of different virulence-related genes was tested by PCR using different primer combinations, as described in Table 1. Uniplex PCR was used for the detection of adhesin genes *icaA, icaB, icaC* and *icaD*[24] and biofilm-associated protein gene *bap, fnbA, aap* and *altE*. Multiplex PCR was used for the detection of accessory regulator genes *agrI, agrII, agrIII* and *agrIV*.[25]

Evaluation of Antibiotic Susceptibility and Detection of Methicillin Resistance

Susceptibility of isolates to different antibiotics was evaluated using the modified Kirby–Bauer method and results were interpreted according to CLSI guidelines.[26] The following antimicrobial agents were tested: cefoxitin (30μg), Teicoplanin (30

Table 1 Summary of the Target and Sequence of the Oligonucleotide Primers Used in the PCR

	Gene	Primer	Nucleotide Sequence	Amplicon Size	Reference
Identification and Methicillin resistance	*SA*	Forward Reverse	5' -AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG-3' 5' -CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA-3'	108 bp	[23]
	SE	Forward Reverse	5' -ATC AAA AAG TTG GCG AAC CTT TTC A-3' 5' -CAA AAG AGC GTG GAG AAA AGT ATCA-3'	124 bp	[23]
	SH	Forward Reverse	5' -GGT CGC TTA GTC GGA ACA AT-3' 5' -CAC GAG CAA TCT CAT CAC CT-3'	270 bp	[23]
	MRS	Forward Reverse	5' -TAG AAA TGA CTG AAC GTC CG-3' 5' -TTG CGA TCA ATG TTA CCG TAG-3'	154 bp	[23]
PIA dependant biofilm formation	*IcaA*	Forward Reverse	5' -TCT CTT GCA GGA GCA ATC AA-3' 5' -TCA GGC ACT AAC ATC CAG CA-3'	188 bp	[24]
	IcaB	Forward Reverse	5' -ATG GCT TAA AGC ACA CGA CGC-3' 5' -TAT CGG CAT CTG GTG TGA CAG-3'	526 bp	[32]
	IcaC	Forward Reverse	5' -ATA AAC TTG AAT TAG TGT ATT-3' 5'-ATATATAAAACTCTCTTAACA-3'	989 bp	[33]
	IcaD	Forward Reverse	5' -ATG GTC AAG CCC AGACAG AG-3' 5' -CGT GTT TTC AAC ATT TAA TGC AA-3'	198 bp	[24]
PIA independent biofilm formation	*Aap*	Forward Reverse	5' -ATA CAA CTG GTG CAG ATG GTT G-3' 5' -GTA GCC GTC CAA GTT TTA CCA G-3'	400 bp	[34]
	AltE	Forward Reverse	5' -CAACTGCTCAACCGAGAACA-3' 5' -TTTGTAGATGTTGTGCCCCA-3'	682 bp	[34]
	FnbA	Forward Reverse	5' -CACAACCAGCAAATATAG-3' 5' -CTGTGTGGTAATCAATGTC-3'	1362 bp	[35]
	Bap	Forward Reverse	5' -GATCCAATTATTGCTGAGCATG-3' 5' -CACCTTCGATATATGGTAGTAAGTC-3'	574 bp	[36]

(*Continued*)

Table 1 (Continued).

	Gene	Primer	Nucleotide Sequence	Amplicon Size	Reference
Typing	*Spa*	Forward Reverse	5' -TAA AGA CGA TCC TTC GGT GAG C-3' 5' -CAG CAG TAG TGC CGT TTG CTT-3'	180–600 bp	[37]
Biofilm regulation	*Agr*	Forward	5' -ATGCACATGGTGCACATGC-3'		[38]
	AgrI	Reverse	5' -GTCACAAGTACTATAAGCTGCGAT-3'	439 bp	[38]
	AgrII	Reverse	5' -TATTACTAATTGAAAAGTGGCCATAGC-3'	572 bp	[38]
	AgrIII	Reverse	5' GTAATGTAATAGCTTGTATAATAATACCCAG-3'	321 bp	[38]
	AgrIV	Reverse	5' -CGATAATGCCGTAATACCCG-3'	657 bp	[38]

μg), Vancomycin (30 μg), Gentamycin (10 μg), Azithromycin (15 μg), Erythromycin (15 μg), Ciprofloxacin (5 μg), Levofloxacin (5 μg), Tetracycline (30 μg), Doxycycline (30 μg), Trimethoprim/sulfamethoxazole (1.25/23.75 μg), Clindamycin (2 μg), Linezolid (30 μg), Chloramphenicol (30 μg), Amoxicillin/clavulanate (10/20μg), Cephalexin (30μg), Ceftazidime (30 μg), Ceftriaxone (30 μg), Meropenem (30 μg), Tobramycin (30 μg), Amikacin (30 μg) and Rifampin (30 μg). Methicillin resistance in Staphylococci was defined as the isolates that showed resistance to cefoxitin and a positive *mecA* PCR test.[27]

Isolates were regarded to be multidrug-resistant (MDR) if they exhibited resistance to three or more antimicrobials that belonged to distinct antibiotic classes and bacterial targets, and extensively drug resistant (XDR) if strains were resistant to at least one agent in all but two or fewer antimicrobial categories (ie, bacterial isolates remain susceptible to only one or two antimicrobial categories).[28]

Staphylococcal Protein a (Spa) Typing

Amplification of the spa gene was performed using the primer pairs shown in Table 1, as described previously.[29] PCR products were sequenced by Macrogen (Korea) and spa types were determined using DNA Gear-A software and the Ridom SpaServer (http://www.spaserver.ridom.de).[30] A clonal complex was defined as a group of at least two related spa types. A spa type that was not part of a clonal complex was considered.[31]

Results

Demographic and Clinical Characteristics

Among the admitted diabetic patients, 58% (n=58/100) were males, 50% were aged 40 years or above and most of them (63%, n=63/100) were illiterate. About half of the cases (55%) were non-smokers. Based on history records, most of the cases had a previous family history, about 49% were diagnosed incidentally and most of them had had the disease for more than 10 years. Hypertension and obesity were commonly reported co-morbidities (37% and 64%, respectively).

The diabetic patients suffered from complications of diabetis such as peripheral neuropathy (55%), retinopathy (33%), peripheral vascular diseases (24%), cerebrovascular diseases, and presence of coronary artery diseases (13% for each) (Table 1).

The analysis of glycemic control using HbA1C showed that 88% of the cases were uncontrolled diabetic patients. Analysis of random blood glucose levels showed that blood glucose levels were above 200 in most cases, which is consistent with HbA1c results (71% of cases had HbA1c values of >9) (Figure 1 and Table 2). Concerning the treatment, the majority of patients (63%) partially managed the disease with insulin and 24% with oral hypoglycemic agents.

The majority of ulcers were neuropathic (48%) and located under the heads of the metatarsal region of the foot (32%). Most ulcers were deep (grade 2 or 3) according to Wagner–Meggitt classification.

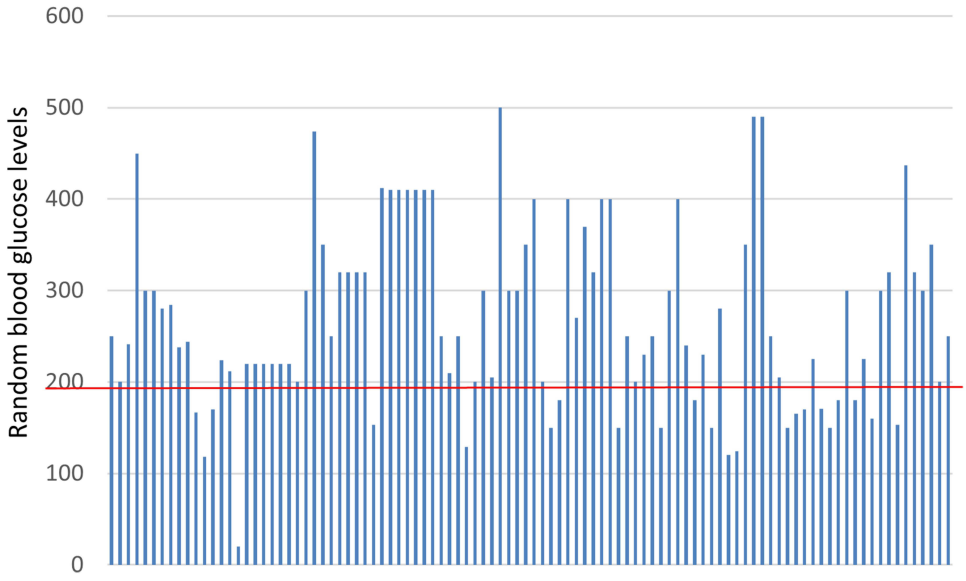

Figure 1 Random blood glucose levels of the cases.

Microbiological Analysis of DFU

Microbiological analysis of the wound swabs showed that 94% of these ulcers were infected, 54% (n=54/100) were polymicrobial, 40% (40/100) were monomicrobial and 6% (n=6/100) were negative. Gram-negative bacteria were detected in 59.49% (n=94/158) of isolates. Gram-positive isolates were detected in 40.5% (n=64/158) of isolates either as mono-infection or coupled with other Gram-negative bacteria. All Gram-positive infected cases contained staphylococcal spp.

Among the 64 Staphylococci isolates, 37.5% (n=24/64) were identified as *S. aureus*, 23.4% (n=15/64) *S. haemolyticus*, and 34.3% (n=22/64) *S. epidermidis*. Interestingly, some samples (n=11/64, 17.1%) were co-infected with more than one type of Staphylococci. six samples showed a co-infection of *S. aureus* and *S. epidermidis*, 3 samples showed co-infection of *S. aureus* and *S. haemolyticus*, and two samples showed mixed infection of *S. epidermidis* and *S. haemolyticus* as shown in Figure 2.

Table 2 Demographic and Clinical Characteristics of Enrolled Patients

Variable	Total Number, N=100 (%)
Age (years)	54.8±12.1
Gender	
Male	58 (58%)
Female	42 (42%)
Smoking status	
Never	55 (55%)
Former	23 (23%)
Current	22 (22%)
Family history of diabetes	
Positive	66 (66%)
Negative	34 (34%)
Duration of diabetes (years), range	14.9±7.3 (1–35)
Co-morbidities	
a) Hypertension	37 (37%)
b) Overweight and Obesity	64 (64%)

(*Continued*)

Table 2 (Continued).

Variable	Total Number, N=100 (%)
Complications of diabetes	
a) Neuropathy	55 (55%)
b) Retinopathy	33 (33%)
c) Peripheral vascular diseases	24 (24%)
d) Cerebrovascular diseases	13 (13%)
e) Cardiovascular diseases	13 (13%)
f) Nephropathy	10 (10%)
Haemoglobin A1C% level:	
a) Controlled (<7)	12 (12%)
b) 7–9	17 (17%)
c) >9	71 (71%)
Treatment	
a) Oral hypoglycaemic agents only	24 (24%)
b) Insulin only	63 (63%)
c) Both	13 (13%)
Type of ulcer	
a) Ischemic	32 (32%)
b) Neuropathic	48 (48%)
c) Mixed	20 (20%)
Ulcer position	
a) Forefoot	16 (16%)
b) Mid foot	17 (17%)
c) Hind foot	21 (21%)
d) Metatarsal head ulcer (MTH)	32 (32%)
e) Big toe	20 (20%)
Depth of ulcer	
Grade 1	27 (27%)
Grade 2	39 (39%)
Grade 3	29 (29%)
Grade 4	4 (4%)
Clinical grading of the DFU	
a) No infection	20 (20%)
b) Mild	38 (38%)
c) Moderate	30 (30%)
d) Severe	12 (12%)

Most Staphylococci were susceptible to linezolid, chloramphenicol, and rifampicin; however, a high level of resistance was observed to amoxiclav, cefoxitin, and oxacillin, as shown in Figure 3. Among the 64 Staphylococci isolates tested, cefoxitin resistance and *mecA* gene were observed in 68.7% (n=44/64) of samples, pointing to the high frequency of methicillin resistance among the isolates.

Screening for Biofilm-Related Genes

Staphylococci were further analyzed for the presence of different virulence-related genes. Most isolates carried *ica*-dependent genes of biofilm formation, where 40.6% (n=26/64) of Staphylococci had *icaA*, 28.1% (n=18/64) had *icaB* and 84.3% (n=54/64) were positive for *icaD*. All isolates were negative for *icaC*. Concerning *ica*-independent genes, spa and *bap* were detected in about 70%, *aap in* 65.6%, *altE* in 20.3%, and *fnbA* in 25%.

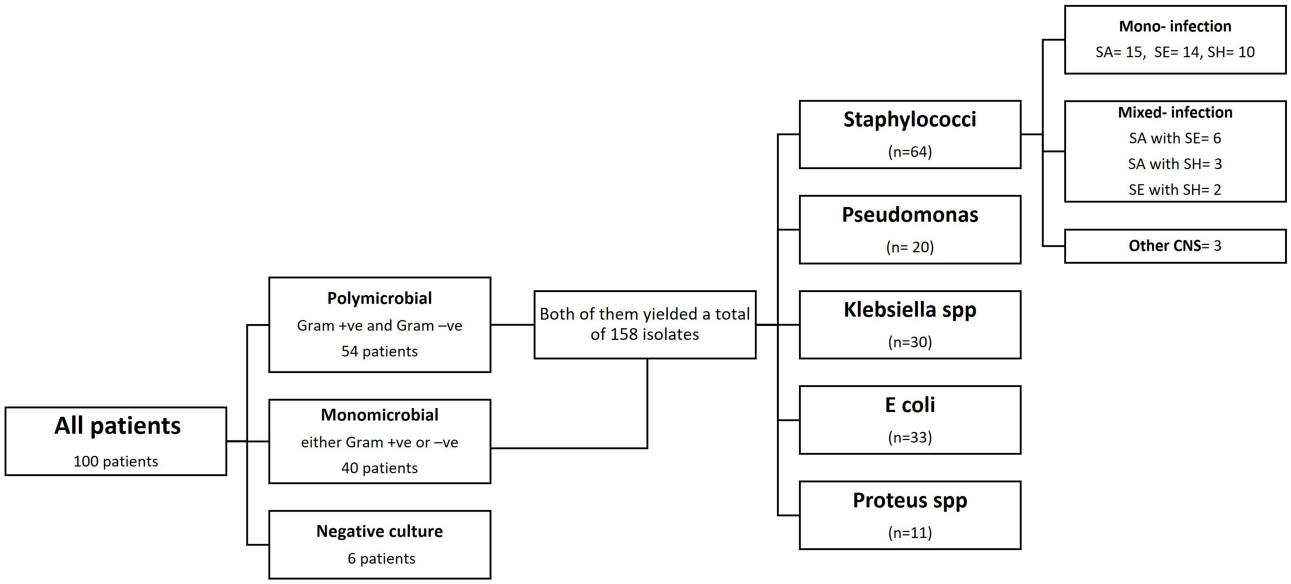

Figure 2 Flow chart showing the microbiological analysis of DFUs.
Abbreviations: SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*; SH, *Staphylococcus haemolyticus*.

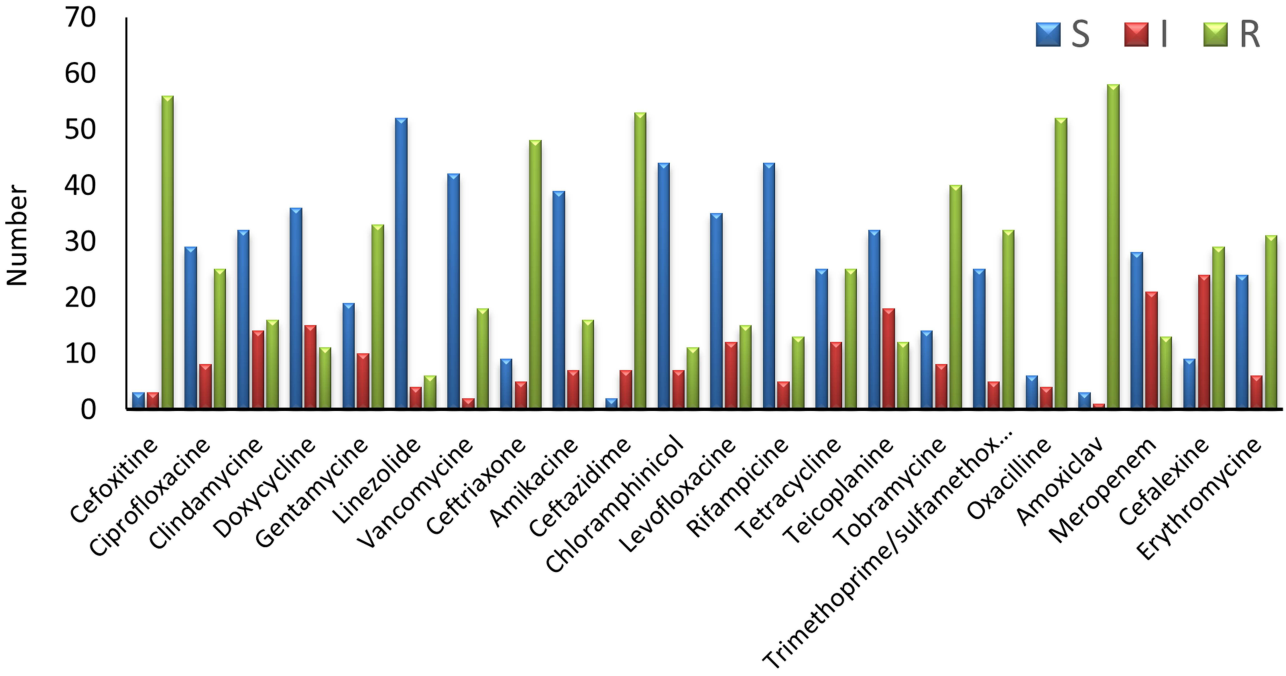

Figure 3 Antibiotic sensitivity pattern of isolated Staphylococci.

The quorum-sensing genes *agrI* and *agrII* were detected in 15.6% (n=10/64) and 18.7% (n=12/64) of the Staphylococci, respectively. Both *agrIII* and *agrIV* were detected in 14% (n=9/64). All isolates infected with *S. aureus* were positive for *spa*.

Correlation Between Strength of Biofilm Formation and Number of Bioflim related Genes

Biofilm formation was assessed by microtiter plate which showed that all Staphylococci were biofilm producers. The majority (59.3%, n=38/64) were strong biofilm producers, 32.8% (n=21/64) were moderate and 7% (n=5/64) were weak biofilm producers. The number of biofilm genes was significantly associated with higher MTP values (p value=0.011), as shown in Table 3.

Table 3 Number of Positive Genes According to Strength of Biofilm

	Strength of Biofilm			P-value
	Strong (n=38)	Intermediate (n=21)	Weak (n=5)	
Absorbance of MTP (mean ± SD)	5.12 ± 1.85	4.05 ± 1.86	3.40 ± 1.14	**0.011***
Predominant genes	*Spa* (88.2%), *icaD* (79.4%), *bap* (70.5%), *aap* (67.6%)	*Spa* (79%), *icaD* (75%), *aap* (50%), *bap* (41%)	*Spa* (80%), *icaD* (60%), *aap* (40%), *bap* (20%)	**NA**

* p-value < 0.05 is considered as significant difference.
Abbreviation: NA, Not applicable.

Distribution of Biofilm-Related Genes Among Isolated Staphylococci

Staphylococci isolates tend to form biofilms in different ways and this can be illustrated by the fact that most samples were positive for more than one gene; 93.7% (n=60/64) were positive to multiple biofilm genes and 6.2% (n=4/64) were positive to one gene. *Spa, icaD, bap and aap* were the most detected genes. Interestingly, the number of biofilm-related genes played an important role in biofilm formation; isolates that were positive to 7 and 9 genes were strong biofilm formers (Figure 4).

Strength of Biofilm Formation is Correlated with Severity of Infection and Type of Ulcer

Biofilm formation is highly prevalent among different types of ulcers. Also, it exacerbates the systemic infection associated with diabetic foot. All severely infected wounds were either strong or intermediate biofilm formers. Weak biofilm formers were only in moderate infections. About 50% (n=19/38) of strong biofilm formers were isolated from mildly infected wounds, 19% from moderate infections, 31% from severely infected ulcers. In addition, 41% of intermediate biofilm formers were isolated from mildly infected ulcers, 29% from wounds clinically evaluted as non-infected, 4% from moderate infections and 26% from severely infected wounds.

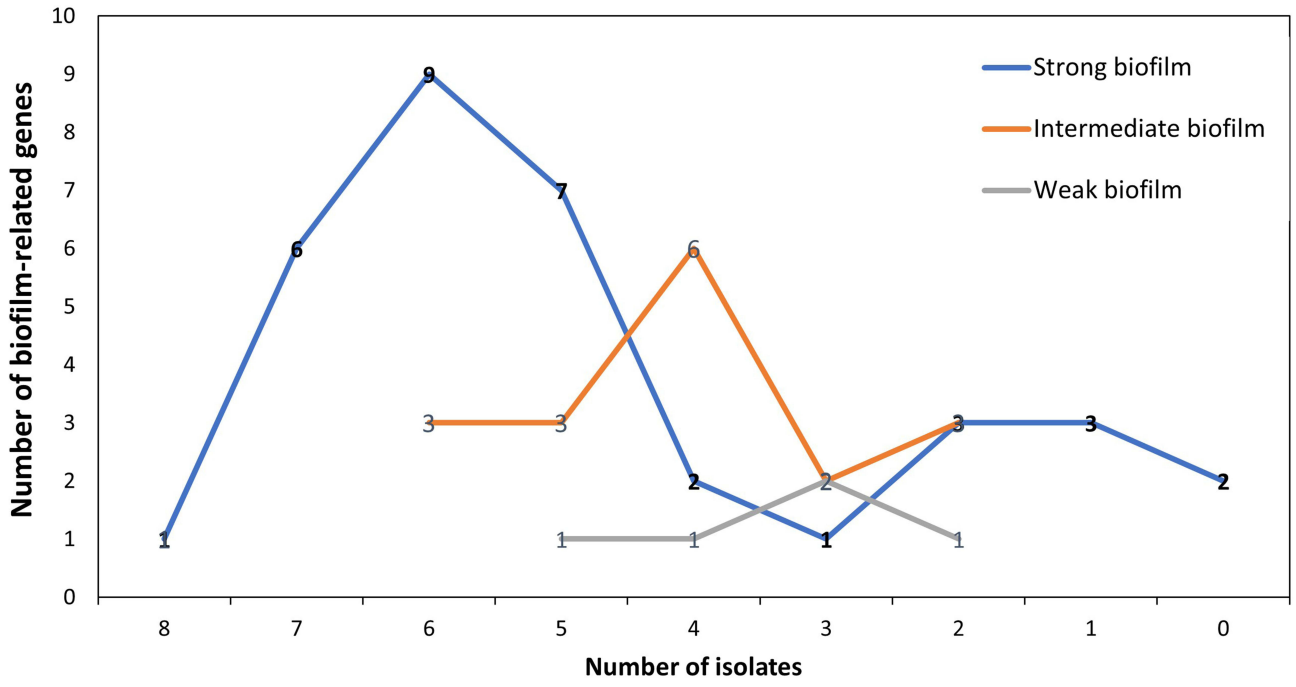

Figure 4 Relation between number of biofilm-associated genes and strength of biofilm.

Table 4 Summary of Spa Genotyping, Resistance Profiles and Biofilm Production

spa Type	agr Type	Resistance Profile	Biofilm Strength
t034	agrII	MRSA	Strong
t068	agrII	MRSA	Strong
t016	agrII	MRSA	Strong
t033	agrII	MRSA	Strong
t031	agrII	MRSA	Strong
t057	agrII	MRSA	Strong
t021	agrII	MRSA	Strong
t012	agrII	MRSA	Strong
t192	agrIII	MRSA	Strong
t023	agrIV	MRSA	Intermediate
t072	Negative	MRSA	Intermediate
t017	Negative	MRSA	Intermediate
t020	Negative	MRSA	Intermediate
t133	Negative	MRSA	Intermediate
t058	Negative	MRSA	Intermediate
t162	Negative	MRSA	Intermediate
t223	Negative	MRSA	Intermediate

Correlation Between Strength of Biofilm Formation and Resistance to Antibiotics

As predicted, there was a direct relation between biofilm formation and drug resistance: 78.9% (n=30/38) of strong biofilm forming isolates were MDR and all XDR were strong biofilm formers.

Results of Spa Typing

Sequencing of spa gene in *S. aureus* isolates showed that our isolates represent a collection of 17 different spa types like t034, t023, t068, t016, t033, t031, t057, t021, t012, t192, t072, t017, t020, t223, t133, t058 and t162. Each of t034, t016, t057, t012 and t021 were detected in 8.3% (n=2/24) while t033 was in 12.5% (n=3/24) of spa type *Staphylococci*. As shown in Table 4, spa types that were strong biofilm formers showed positive results for agr genes, mostly for agrII, and were resistant virulent isolates. There was no predominant type, probably because samples were from outpatient clinics.

Discussion

Diabetic foot ulcers are an important cause of increased mortality and morbidity, particularly when coupled with pathogenic microorganisms such as *S. aureus* due to a high load of different virulence factors especially biofilm formation. One of the main problems of bacterial biofilms is the associated resistance to a wide variety of antibiotics.[39]

In this investigational study, a strong relationship between uncontrolled HA1c, severity of infection and strength of biofilm formation was observed. About 14.7% of strong biofilm forming strains belonged to patients with uncontrolled diabetes as their HA1C was 12–19% and had severely infected diabetic foot. Of strong biofilm isolates, 55% were mildly infected diabetic foot and A1C was 6.5–15. Callahan et al[40] highlighted the role of uncontrolled HA1c as a risk factor for increasing risk of infected diabetic foot.

Regarding type of ulcer, we found that neuropathic ulcers accounted for 48%, ischemic ulcers for 32% and neuroischemic (mixed) ulcers for 20%. These percentages were consistent with studies that were done in the MENA region,[41] which showed that neuropathic ulcers accounted for 36% and ischemic ulcers for 12%. Another study, by Petropoulos et al,[42] showed that Egypt has the second highest percentage of neuropathic ulcers (61.3%) in the MENA region.

Approximately 7% of diabetic patients had heel ulcers. Similar to our findings, a larger cohort of patients with foot ulcers found that 10% of all foot ulcers were located in the heel.[43] In clinical practice, heel ulcers are usually regarded as poorly healing ulcers mainly due to reduced blood supply, and researchers have found that these ulcers heal more slowly than those on the metatarsophalangeal joint or the toes.[44]

Our Staphylococcus isolates were mostly sensitive to linezolid (80%) and this was similar to a study performed by Abubakar et al.[45] Limited use of rifampicin, used mostly in cases of tuberculosis, and clindamycin in treatment of cases of diabetic foot infections in Egypt is a factor explaining their relative sensitivity pattern.

In our study we have 15 (23.4%) *S. haemolyticus* isolates either alone or mixed with other Staphylococcus species like *S. aureus* and *S. epidermidis*. Although, *S. haemolyticus* is a less common species of Staphylococcus, its prevalence was relatively high. Similar findings were reported by other studies.[46,47] However, other studies, such as that carried out by Mottola et al,[48] showed a lower percentage (1.8%) of S. haemolyticus. Wang et al[49] reported that 2.5% of *S. haemolyticus* were isolated from diabetic foot ulcers. Ge et al[50] isolated *S. haemolyticus* at a percentage of 9% from diabetic foot ulcers. This difference in percentages may be due to the variation in sample types, number of studied population and geographical locations, but it pointed to the importance of atypical microorganisms that contributed to infection of the diabetic foot ulcers. Another important factor for limited isolation of *S. haemolyticus* is the misidentification of mannitol salt agar because both *Staphylococcus aureus* and *S. haemolyticus* cause fermentation of mannitol and produce yellow colonies.[50,51]

Staphylococcal isolates were mainly strong and moderate biofilm formers (59.3% strong, 32.8% moderate and 7% weak). Our data were in line with Tiwari et al[52] whereby isolated Staphylococci were from chronic wounds and formed mainly strong and moderate biofilms. Another study in Egypt, by Gad et al,[53] reported that 51.4% of biofilms were strong, 37.1% were moderate, and 11.4% were weak. A lower rate of biofilm formation was demonstrated by Neopane et al[54] and their biofilm strength results were 6.97% strong, 27.9% moderate and 34.88% weak. Variation may be due to different types of samples, presence of foreign bodies, different growth conditions and the use of various sugar supplementation for biofilm formation in Staphylococci.

Concerning the distriubtuion of biofilm-related genes among staphylococci, Diemond-Hernández et al[33] reported *icaA* in 10.3% and *icaD* in 97.5% of *S. aureus* isolates and did not detect *icaB* or *icaC*. Mirzaee et al[55] showed higher percentages than our study. Torlak et al[56] reported high prevalence of *ica* genes among *S. aureus* where all isolates were reported to possess *icaA* and icaD genes. Arciola et al[24] and Gad et al[53] detected *icaA* and *icaD* genes in all biofilm-forming *S. aureus* isolates. The discrepancy between the results of different studies may be caused by the diversity of bacterial genetic characterization, source of isolation, and environmental factors.

In our study, 8 isolates (12.5%) were negative to *ica* (*A, B, C and D*) genes. However, these isolates were found to be biofilm formers phenotypically by MTP method. One of them was a weak biofilm former, one was an intermediate biofilm former and 6 were strong biofilm formers. This was in line with results of Mirzaee et al[55] who reported a *S. aureus* isolate that is negative for all *ica* genes but still produced biofilm. Therefore, our study includes both *ica* genes and genes responsible for biofilm formation using *ica*-independent method. On the other hand, inability of biofilm formation in some Staphylococcal strains, despite the presence of *ica* genes, can be caused by insertion of a 1332-bp insertion element causing its inactivation.[57] Our results were in agreement with Vindel et al[58] that showed *agrII* is the most prevalent agr gene followed by *agrI* (14.8%), *agrIII* (3%) and *agrIV* (0%). *agr* deficiency represents an adaptive approach allowing the microorganism to avoid host immune system and to promote colonization.[59] In fact, the majority of *agr*-deficient strains have been linked to biofilm formation.[60] In line with our results, 55.8% of *agr*-negative strains were strong biofilm formers, 38.2% were intermediate and 5.8% were weak.

In an *ica*-deletion mutant of *S. aureus* strain protein A (*spa*) production was found essential for biofilm formation.[61] Most strong biofilm formers were positive to 4–8 genes. However, 2 (5.2%) of strong biofilm former isolates were

positive only to spa gene and were found to be strong biofilm formers. Moreover, 5 (13.1%) of our samples were positive for only 2 genes and were found to be strong biofilm formers. in these samles spa wasone of these two positive genes, and the other gene was *agrI*, *bap* or *icaD*. Our results showed that spa is predominant in strong and moderate biofilm forming isolates. Exogenous spa was able to restore biofilm formation in spa mutants, indicating that spa does not need to be covalently attached to the cell wall.[61] Spa-negative strains were found to form biofilm but were also negative for other tested genes in our study, which could be explained by the role of other biofilm genes not tested in our study. Some studies have linked the absence of spa protein to point mutations.[62] Hospital-associated (HA) MRSA are typically multidrug resistant; in our study they mainly belong to *agr* types I or II and carry *mecA* gene in many types of spa like t034, t023, t068, t016, t033, t031, t057, t021, t012, t192, t072, t017, t020, t223, t133, t058 and t162. This was similarly reported by Monecke et al[63] and Ćirković et al.[64]

In our study, t034 spa type was detected. This genotype carried the *mecA* gene, was vancomycin sensitive, and produced strong biofilms, as similarly reported by Köck et al[65] t034 is considered to be the most widely distributed spa type in Oman.[66] Spa types t012 and t021 have been previously isolated from wound infections[13] and were HA MRSAe. Our study declared that isolates which belonged to spa t016 were *agrII* type and were MRSA, and strong biofilm formers, while the study of Goudarzi et al[67] reported that t016 isolates belonged to *agrI*. Spa t031 has been isolated in MENA region from nasal swabs and reported by Verhoeven et al.[68] In our study, it was a strong biofilm former and belonged to *agrII* group. Von Eiff et al[69] detected MRSA strains of spa types t033 and t057 circulating in Germany and other parts of Europe on the basis of several well-characterized *S. aureus* strain collections. We have isolated both types from our *S. aureus* samples. t033 is considered the most widely distributed type in the Netherlands.[70] Spa type t068 was isolated in the Netherlands[71] from bloodstream samples and its presence was associated with bacteraemia and it had TSST.

Other spa types such as t017, t020, t023, t058, t072, t133, t162, t192 and t223 were intermediate biofilm formers and were negative for *agr* genes. t017 was also detected in the UK.[72] t020 was found in Scotland using high-resolution melting analysis.[72] t023 was first discovered in Germany[73] and t223 is the most predominant type in Turkey.[70]

In conclusion, infection of diabetic foot ulcers is a serious medical situation. The majority of DFUs is polymicrobial. S. aureus and other coagulase-negative staphylococci are major contributors to infected DFUs. MDR and biofilm formation are marked among isolates. The severity of DFU is directly related to the number of biofilm genes. Therefore, cleaning the wounds, monitoring for signs of infection, and receiving proper antimicrobial therapy are extremely important measures to avoid further complications from MDR and biofilm-forming bacteria.

Abbreviations

DFU, diabetic foot ulcers; *S. aureus*, *Staphylococcus aureus*; MRSA, methicillin resistant *Staphylococcus aureus*.

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Disclosure

The authors declare no conflicts of interest in this work.

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