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RESEARCH ARTICLE

Phenotypic and Genotypic Detection of Biofilm Formation in Methicillin-Resistant Staphylococcus Aureus Isolated from Diabetic Foot Infections

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ARTICLE INFO	ABSTRACT
  *Corresponding Author: pstm.abbas.h.habib@jmu.edu.iq	Background: Methicillin-resistant S. aureus biofilm formation is a common isolate in diabetic foot infections; biofilm development contributes significantly to the pathophysiology and reduces antibiotic sensitivity, making treatment challenging. Objectives: To identify methicillin-resistant S. aureus in infected diabetic feet, their antibiotic susceptibility, and their phenotypic and genotypic capacity for biofilm formation. Materials and methods: A cross-sectional study was conducted from August 2023 to January 2024 at Al-Manathira General Hospital in the Najaf, Iraq. Patients with diabetic foot infections were enrolled in the study. Data regarding the age, sex, residence, antibiotic treatment, smoking, and past medical history were recorded for each participant. The disk diffusion technique was used to determine the existence of methicillin resistance. Cefoxitin disks were used for this purpose. The antibiotic susceptibility of the isolates was tested by the VITEK® 2 system. Biofilm production was assessed using the microtiter plate method. Polymerase chain reaction was used to identify the presence of the icaD gene in the bacterial strains. Results: Out of 150 patient samples, there were 102 S. aureus isolates (93.1% were MRSA, and 6.9% MSSA). Many S. aureus isolates demonstrated resistance to beta-lactam antibiotics and fusidic acid. In contrast, a smaller percentage of MRSA isolates resisted vancomycin and trimethoprim/sulfamethoxazole. No isolates were resistant to linezolid or tigecycline. All MRSA isolates produced biofilm with varying degrees: strong 27.4%, moderate 41.2%, and weak 31.4% respectively. Detection of the icaD gene, signifying biofilm-forming capability, was remarkably high (90.19%) among MRSA isolates. Conclusions: This study indicated that methicillin-resistant isolates developed more biofilms and were highly resistant to most drugs. The high prevalence of the biofilm icaD gene in multidrug-resistant S. aureus underscores the need for a thorough knowledge of its epidemiology, molecular study, and effective biofilm therapy for S. aureus infection.

INTRODUCTION

Diabetic foot ulcers (DFUs) are a complex condition affecting the lower limb amputations and disability of diabetic patients. It encompasses a combination of nerve damage (neurologic abnormalities), impaired blood circulation (peripheral vascular disease), and metabolic complications of diabetes, all of which can contribute to foot infections, ulcer formation, and even tissue destruction [1].

S. aureus, including methicillin-resistant S. aureus (MRSA), is among the top threats to global public health and is one of the main bacterial isolates in diabetic foot infections. MRSA infections are difficult to eradicate since these strains are often multidrug-resistant, and increasing the problem level as well as MRSA can form biofilms on biotic and abiotic surfaces [2]. Biofilms form when bacterial cells aggregate in complex communities embedded in bacterial-produced extracellular polymeric substances attached to each other and a surface. These extracellular substances are composed of exopolysaccharides (EPSs), proteins, and other macromolecules, such as extracellular DNA [2].

Biofilms can form on medical implants or tissue surfaces to protect pathogenic bacteria from immune-detective mechanisms and antibiotic killing [3]. MRSA has evolved to increase biofilm formation in response to selective pressures within healthcare and community settings. Staphylococci, including MRSA, are known to be the most common cause of biofilm-associated infections; they contribute to more than 80% of all MRSA infections in humans [4]. MRSA strains produce penicillin-binding protein (PBP) with decreased affinity. The mecA gene, located on the Staphylococcal cassette chromosome mec (SCCmec) mobile genetic element (MGE), encodes this protein [5].

S. aureus is armed with a battery of virulence factors, including panton-valentine leukocidin(PVL), exfoliative toxins, toxic shock syndrome toxin-1(TSST-1), and staphylococcal enterotoxins. Enzymes (Coagulase, Hyaluronidase, Proteases, DNases, RNases, lipases, hemolysins, nucleases, penicillinase, and capsule. S. aureus has numerous surface proteins named microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that mediate bacterial adherence to the extracellular matrix of the host and play a key role in the initiation of infections. Furthermore, these proteins contribute to biofilm formation and evading the immune system, in addition to the ica operon that produces the polysaccharide intercellular adhesion (PIA) an important factor in biofilm formation [5]. Staphylococcal protein A (SpA), a molecule that cross-links B-cell receptors, is both necessary and sufficient for this immune evasion mechanism. Additionally, SpA must firmly adhere to bacterial peptidoglycan, while CD4 T-cells aid in regulating the immune system responses of infected hosts [6].

Biofilm-forming S. aureus may delay the growth of new epithelial layers in infected tissues, resulting in prolonged healing time. Moreover, biofilm activity on S. aureus increases the resistance level to host immune defenses and the resistance pattern to different antibiotics [7]. This high antibiotic resistance can be attributed to limiting the diffusion rate, as in oxacillin and vancomycin, reducing the growth rate and diminishing the uptake of antibiotic molecules, preventing antibiotics from reaching their specific targets like fluoroquinolones, antibiotic inactivation by the extracellular matrix, and the occurrence of horizontal gene transfer and the exchange of plasmid-mediated resistance genes [2].

Four major stages are involved in the formation of biofilms: first, initial attachment; this stage is mediated mainly through the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs); second, biofilm accumulation forming multiple bacterial layers; and third, biofilm maturation with the production of polysaccharide-intercellular adhesins (PIA) synthesized by the products of the icaADBC operon or by S. aureus surface proteins such as surface-anchored proteins C and G (sasC, sasG), biofilm-associated protein (Bap), collagen-binding protein (cna), fibronectin-

binding proteins A and B (fnbA, fnbB), and fibrinogen-binding protein (fib). The fourth stage is the dispersal of cells in the planktonic state from mature biofilms to spread to new sites elsewhere, mediated by quorum sensing, especially the accessory gene regulator agr [8].

MRSA's resistance to common antibiotics is a critical challenge in diabetic foot infection treatment. Also, biofilm formation further complicates treatment by making the bacteria less susceptible to antibiotics. The participation of biofilm formation in clinical isolates of S. aureus has received increasing attention. Since there is a correlation between biofilms and chronic infections and resistance to antimicrobial agents, it is still essential to extend the research on the genes that modulate biofilm formation, antibiotic resistance, and associations with S. aureus infections.

With the expanding number of diabetic patients, it is becoming increasingly challenging to manage diabetic foot infections, which represent the most common complication with a high risk of mortality and morbidity owing to the factors of peripheral neuropathy, ischemia, and dysregulation of immune infiltration. Diabetic wounds are often prone to bacterial infection and biofilm formation. Identifying MRSA virulence and treatment strategies and understanding the biofilm-forming properties of MRSA in diabetic foot infections may aid in developing more effective treatment strategies for these challenging infections.

To our best knowledge, in the Al-Najaf province, there are not enough data on the prevalence of biofilm-forming MRSA isolates and their antibiotic susceptibility patterns in health institutes, increasing the number of diabetic patients with infected diabetic feet and therapy failure. Hence, the current study was carried out to investigate the prevalence of antibiotic resistance and biofilm-forming MRSA isolates using both phenotypic and genotypic methods.

RESEARCH METHOD

Study Design & Population Sample Collection

A cross-sectional study was conducted from August 2023 to January 2024 at Al-Manathira General Hospital in Al-Najaf, Iraq. One hundred fifty samples were collected from patients suffering from diabetic foot infections.

Patients with diabetes mellitus (types 1 and 2) referred to the diabetic foot unit suffering from infected diabetic feet as diagnosed by the physician were enrolled in the study. Outpatients or before being treated with antibiotics were the inclusion criteria. While, patients who took any antibiotics in the last seven days, subjected to surgery, dialysis, and hospitalization, or other sites infectious conditions were excluded from the current study. Also, all staff in the hospital setting were excluded from the study. The Ethical Committee of Jaber ibn Hayyan University of Medical and Pharmaceutical Sciences approved the study after obtaining informed consent from each participant dated March 2, 2024, with reference number 1276.

Data concerning the age, sex, residence, history of antibiotic taking, type of treatment (oral hypoglycemic agent or insulin), smocking, and other medical diseases (hypertension or heart attack) from each participant were recorded. Aime's transport medium was used for immersing sterile cotton swabs before transporting them directly to the laboratory.

Cultivation and Isolation Bacteria

Each diabetic foot infection swab sample was cultured first on Mannitol Salt Agar to determine the difference between gram-positive and gram-negative bacteria at 37°C for 18–24 hours.

Identification and Antibiotic Susceptibility Testing

The identification and antimicrobial resistance (minimum inhibitory concentrations) of S. aureus were tested by using a fully automated VITEK® 2 compact system Gram-positive ID card and an AST

card. The procedure of work was according to the standard operating procedure for the VITEK® 2 compact system. AST data were interpreted using the Clinical and Laboratory Standards Institute (CLSI) standards according to the manufacturer's instructions from BioMérieux, France, and the Advanced Expert System [9]. The isolates' susceptibility was evaluated with 17 antibiotics using the VITEK® 2 compact system using following antibiotics: cefoxitin screen OXSF 30 μg, benzylpenicillin P 10 μg, oxacillin OX 1 μg, gentamicin GEN 10 μg, ciprofloxacin CIP 5 μg, moxifloxacin MXF 30 μg, inducible clindamycin resistance ICR 0.5 μg, erythromycin E 15 μg, clindamycin TE 2 μg, linezolid LZ 30 μg, teicoplanin TEI 30 μg, vancomycin VA 30 μg, tetracycline TC 30 μg, tigecycline TGC 15 μg, fusidic Acid FA 10 μg, rifampicin R 5 μg, trimethoprim/sulfamethoxazole SXT 1.25/23.75 μg.

Detection of Methicillin-Resistant S. Aureus (MRSA)

This study identified methicillin-resistant S. aureus (MRSA) using a disk diffusion test with cefoxitin. The disk diffusion test defined resistance based on cefoxitin inhibition zones not exceeding 21mm for the tested strains [9].

Biofilm Formation Test

Phenotypic assay

Congo red agar method

A simple qualitative method was used for detecting biofilm-forming bacteria based on colony color changes in congo red agar [10]. Biofilm formation was assessed by inoculating brain heart infusion broth (37g/L) (Himedia/India) supplemented with Congo red (Himedia/India) (0.8 g/L) and glucose (36 g/L), followed by incubation at 37°C for 24 hours and observing colony color changes. Biofilm formation shows dark, dry, crystalline colonies, while non-producers form pink colonies [11].

Inoculated newly cultivated isolates were placed in 10 ml of brain heart infusion (BHI) broth with 1% glucose and incubated at 37°C for 24 hours. A sterile polystyrene microtiter plate (MTP) method with ninety-six flat bottom drills was filled with 200 milliliters of the prepared bacterial solution. Also, control organisms were placed in MTP. To maintain sterility and distinguish non-specific binding, sterile broth was exclusively employed. After 24 hours of incubation at 37°C, the plate was carefully tapped to eliminate the contents of the wells and washed twice with 200 μl of phosphate-buffered saline. The washing was done four times to eradicate any free bacteria from the wells. To fix the biofilms produced as bacteria adhered to the wells, sodium acetate (2%) was put into the wells and allowed to sit for 30 minutes. Crystal violet (0.1%) was used to stain the fixed biofilms. The wells were carefully cleansed with deionized water after 30 minutes to eliminate any remaining stains. The MTP was then incubated at 37 °C for roughly 30 minutes to obtain total dryness. Then, 200 μl of 99% ethanol was added for approximately 10 minutes. Finally, the biofilm concentration in every well was determined by measuring the optical density at 630 nm with an ELISA microtiter plate reader. The examination was performed three times, with a mean of three optical density (OD) values obtained. The optical density measurements showed bacterial adhesion to the wells and the formation of biofilms. As in previous research, the optical density values were calculated, and biofilm formation was classified as weak, moderate, and strong based on the optical density (OD) value. OD values less than 0.120 indicate weak biofilm formation, while values between 0.120 and 0.240 indicate moderate biofilm formation. Values greater than 0.240 indicate strong biofilm formation [12].

2- Genotypic assay detection of the *icaD* gene by PCR

The monoplex PCR technique is employing a Promega master mix targeted the icaD gene in S. aureus isolates designed by Macrogen, Korea. The provided information details the primer sequences used to amplify the icaD gene, which is associated with biofilm formation in bacteria, using a technique called PCR. Gene target: icaD, PCR primers: forward primer (icaD-F): 5' TGGTCAAGCCCAGACAGAGG3', reverse primer (icaD-R): 5' TGATAATCGCGAAAATGCCC3', amplicon

size: 242 base pairs. The experiment was sustained according to the following program: initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 45 seconds, and a final extension at 72°C for 30 seconds.

DNA Extraction

DNA was extracted from the broth sample using genomic DNA and purified using a purification kit (Promega, USA) for icaD gene isolates. PCR was used to identify the primer described above. The primer-detecting icaD gene was designed by Macrogen, Korea [1]. The extracted DNA was stored at -20°C until used. A nanodrop spectrophotometer is used to estimate the concentration and purity of DNA samples.

Statistical Analysis

Data were presented in an Excel 2016 sheet and analyzed using the software Statistical Package for the Social Sciences (SPSS), version 26. The results were presented in tables as frequencies and percentages. The chi-square test evaluates the association between any two categorical variables. The level of significance was defined as a P-value of less than 0.05, which would be considered statistically significant. Additionally, use the mean and standard deviation

DATA ANALYSIS

The study examined the demographic and clinical characteristics of patients with infected diabetic feet, as shown in Table 1.

Table 1. Demographic and clinical characteristics of the participants (N = 150).

Variables	Category	NO. (%)
Sex	Males	112 (74.7%)
	Females	38 (25.3%)
Age groups (years)	45-54	75 (50%)
	55-64	48 (32%)
	65-74	24 (16%)
	75-85	3 (2%)
	Mean±SD	37.5±24.03
Residence	Rural	93 (62%)
	Urban	57 (38%)
Smoking	Yes	89 (59.3%)
	No	61 (40.7%)
Hypertension	Yes	91 (60.7%)
	No	59 (39.3%)
Heart attack	Yes	8 (5.3%)
	No	142 (94.7%)
Diabetes mellitus types	DMT1	68 (45.3%)
	DMT2	82 (54.7%)
	Oral hypoglycemic agent	79 (52.7%)

	Insulin	54 (36%)
Treatment	Both	17 (11.3%)

Out of 150 swabs collected from patients with diabetic foot, results revealed 133 (88.7%) were detected as Gram-positive (G+ve), and 17 (11.3%) were detected as Gram-negative (G-ve) bacteria. S. aureus was the significantly higher isolated bacterial species in this study (p = 0.001), with a percentage of 102 (68%). Other species of Gram-positive bacteria were 31 (20.7%) isolates, represented by 19 (13%) S. epidermidis and 12 (8%) Enterococcus feacalis. The most common Gram-negative bacilli were Pseudomason aeruginosa (17) (11%).

The Vitek® 2 compact system was used to determine the susceptibility of 102 S. aureus isolates to 17 antibiotics, as shown in Table 2.

Table 2. shows the antimicrobial susceptibility test of S. aureus isolates.

Antibacterial agent	*S. aureus* (n = 102)	
	Resistance (%)	Sensitivity (%)
Cefoxitin Screen	95 (93.1%)	7 (6.9%)
Benzylpenicillin	102 (100%)	0
Oxacillin	102 (100%)	0
Gentamicin	41 (40%)	61 (60%)
Ciprofloxacin	56 (55%)	46 (45%)
Moxifloxacin	56 (55%)	46 (45%)
Inducible Clindamycin Resistance	3 (3%)	99 (97%)
Erythromycin	61 (60%)	41 (40%)
Clindamycin	31 (30%)	71 (70%)
Linezolid	0	102 (100%)
Teicoplanin	8 (8%)	94 (92%)
Vancomycin	2 (2%)	100 (98%)
Tetracycline	40 (39%)	60 (61%)

Tigecycline	0	102 (100%)
Fusidic Acid	87 (85%)	15 (15%)
Rifampicin	3 (3%)	99 (97%)
Trimethoprim/Sulfamethoxazole	2 (2%)	100 (98%)

Detection of Methicillin-Resistant *S. aureus* (MR*SA*)

In compliance with the standards issued by the Clinical and Laboratory Standards Institute (2020), the findings indicated that of the 102 isolates of S. aureus, 93.1% (95) exhibited resistance to cefoxitin antibiotics as shown in Figure 1.
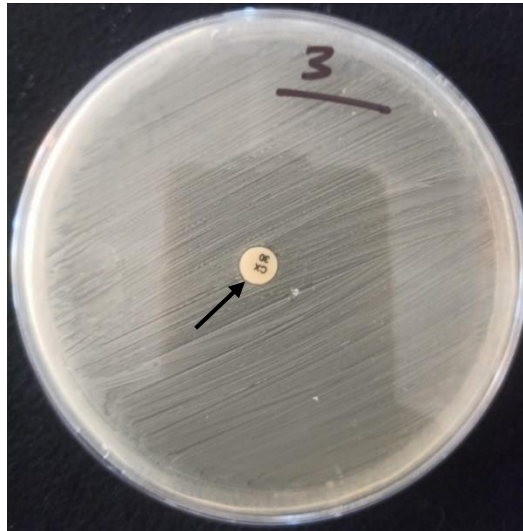

Figure 1. demonstrates the detection of methicillin-resistant *S. aureus* isolates on the Mueller-Hinton agar plate using the cefoxitin disk diffusion method. the arrow shows bacterial growth around the cefoxitin 30 µg disk (high resistance to cefoxitin) located inhibition zone of a round disk, calculated inhibition zone n = 0 according to guidelines (CLSI, 2020).

Biofilm Formation Methods

Phenotypic method

The present study revealed that all 102 (100%) of the isolates were biofilm-forming, as evidenced by the change in color of the red medium at the site of bacterial colony growth after incubation for 24 hours from the color red to black. The method utilized is qualitative, and the medium develops black because the medium contains sucrose, which induces the growth of a biofilm that combines in a process with Congo red dye, causing the color of the medium to transform into black, as seen in Figure 2.
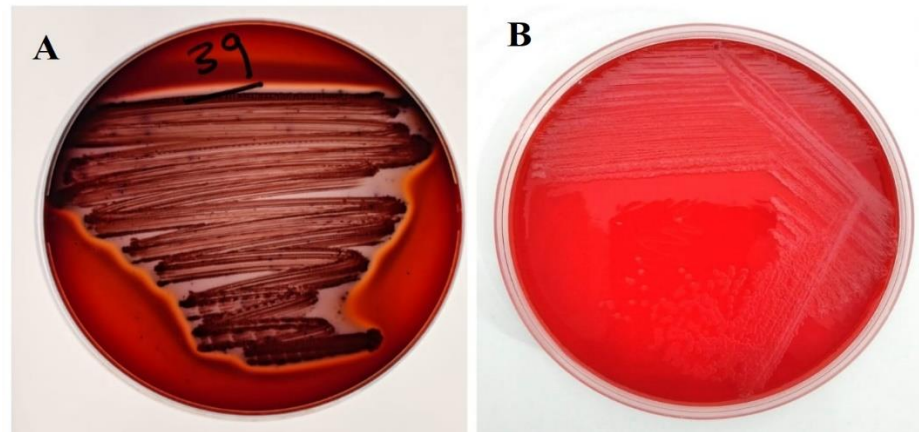
Figure 2. Screening of biofilm producers by congo red agar medium (A) *S. aureus* **(positive control) producing black colonies, (B)** *Staphylococcus epidermidis* **(negative control) producing pink colonies.**

The microtiter plate was utilized to evaluate the capacity of S. aureus strains for biofilm formation. The isolates were classified into three distinct categories based on their biofilm formation capacity, as shown in Table 3. All S. aureus strains included in this study (95 MRSA and 7 MSSA) were capable of forming biofilms. Of the 102 S. aureus strains examined, 100% demonstrated biofilm formation. Within this group, 28 (27.4%) produced strong biofilms, 32 (31.4%) were weak, and 42 (41.02%) were moderate, as shown in Figure 3. There is a highly statistically significant relationship between the level of biofilms and S. aureus isolates (P-value = 0.002), as demonstrated in Table 3.

Table 3. Microtiter plate (MTP) method evaluation of S. aureus isolates for biofilm formation.

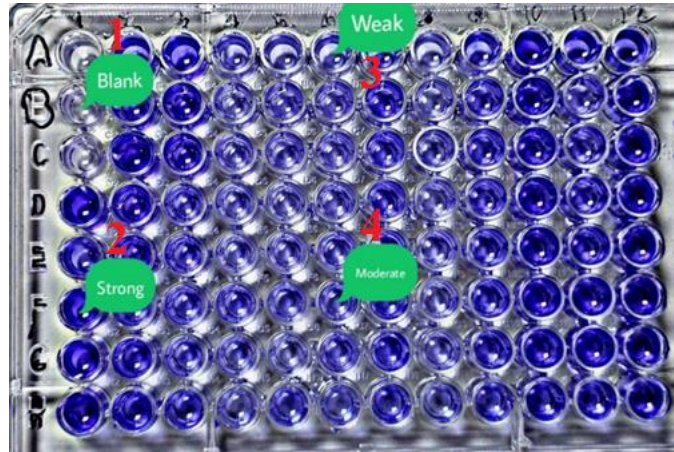| Biofilm formation degree	Frequency *S. aureus* isolates	Percentage %	Mean±SD	P-value
Strong	28	27.4%		
Moderate	42	41.02%		
Weak	32	31.4	34±7.21	0.002
Total	102	100%		

Figure 3. Classification of biofilm-producing *S. aureus* isolates using the MTP method. NO. 1: negative control (without inoculation); NO. 2: strong biofilm producer; NO. 3: weak biofilm producer; and NO. 4: moderate biofilm producer.

Genotypic method of detection of the *icaD* gene by PCR

The detection of the ica operon in the tested strains was confirmed by amplification with PCR of a specific 242-bp segment of the icaD gene as shown in Figure 4. The correlation between biofilm formation and the icaD gene was then investigated. The high icaD gene prevalence among S. aureus isolates is a significant finding. The icaD gene occurs frequently in S. aureus isolates. As expected, a significant percentage of S. aureus isolates, 92 (90.19%), tested positive for the icaD gene. S. aureus isolates, there is a significant correlation between the detection of the icaD gene production genotype and the biofilm production phenotype.
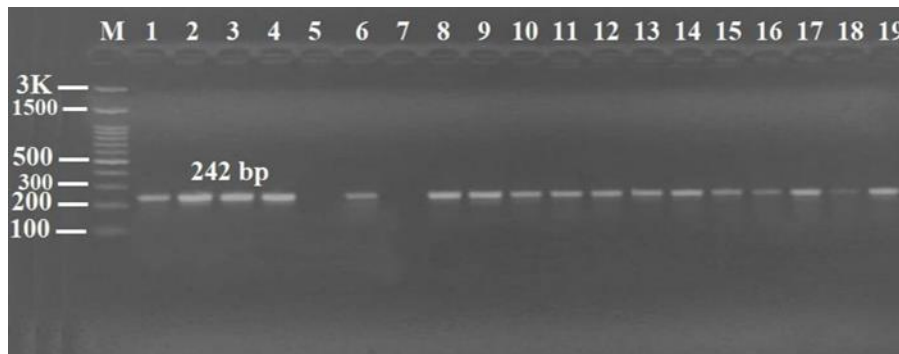

Figure 4. demonstrates an agarose gel electrophoresis image of PCR amplification for the biofilm-associated icaD gene in S. aureus isolates. Lane M represents the DNA ladder (100 bp), whereas lanes 1, 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, and 19 confirm the presence of the icaD gene in a significant portion of the analyzed isolates.

DISCUSSION

According to the demographic and clinical characteristics of the participants. The study's results revealed a higher infection rate in male patients compared to female patients, potentially due to the increased exposure of men in Iraq to environmental factors [1]. Male predominance in DFI may be linked to factors such as gender-related differences in lifestyles and professional roles that require the feet to tolerate more pressure as a result of work, an increased level of outdoor work, and poor compliance with foot care practices. Females heal wounds more quickly than males, which could be attributed to hormonal differences. The increased estrogen receptor in females promotes wound

healing, acting as endogenous enhancers of healing processes. Conversely, in males, the increased level of androgen is considered detrimental to wound healing, as androgenic species reduce dermis repair [13].

Age was a risk factor for amputation, peripheral vascular disease (PVD), and neuropathy among diabetic patients. With their increasing ages and disease course, elderly patients with diabetes frequently also have peripheral neuropathy and vascular lesions, leading to diabetic foot ulcers that poorly heal over a long treatment period [13].

Other lifestyle factors smoking is an important risk factor for diabetes. At the beginning of the 21st century, a sample survey in China revealed that up to 70% of men smoked. The prevalence of diabetes in smokers is approximately 20% higher compared with nonsmokers, with a higher risk for those who started smoking earlier and those who have smoked longer. Older people are more likely to be at risk of developing diabetes [14].

The rise in type 2 diabetes is associated with lifestyle factors like obesity, aging, an inadequate diet, and physical inactivity, all of which contribute to the increase in type 2 diabetes. These are becoming more prevalent as a result of variables such as a Westernized diet and decreased physical activity. A person living with T2DM does not produce enough insulin (insulin deficiency) or has body cells that are not able to use hormones properly (insulin resistance). The pancreatic cells produce insulin, a hormone that regulates blood sugar levels. Genetic factors, obesity, a sedentary lifestyle, and age all contribute to insulin resistance. At first, the body tries to make up for insulin resistance by producing more insulin, but eventually, it can't keep up, leading to high blood sugar and type 2 diabetes [15]. Oral medications are likely the first line of treatment for many type 2 diabetics in Iraq, especially for those with good blood sugar control. Insulin becomes necessary when oral medications aren't sufficient to manage blood sugar levels effectively. In Iraq, oral therapy treats approximately 70% of T2DM patients, while injectable medications treat 30% [16].

Several previous studies have shown that diabetic foot infections contain polymicrobial infections such as S. aureus, Streptococcus, and Pseudomonas, with S. aureus being the most common [1]. The study revealed that S. aureus was the most common gram-positive bacteria found in diabetic foot infections. It was followed by S. epidermidis, Enterococcus feacalis, and Pseudomonas aeruginosa. This is alarming since all patients were diabetic, This is alarming since all patients were diabetic, and this may put a patient at Angiopathy and neuropathy are the main predisposing factors of diabetic foot infections (DFIs), together with muscular atrophy and extrinsic triggers, such as trauma, in the presence of abnormal immunity and ischemia as aggravating factors. These factors collectively result in the loss of skin integrity, favoring the development of DFIs [17].

There is a high rate of bacterial resistance to common beta-lactam antibiotics like benzylpenicillin, oxacillin, and cefoxitin, highlighting the worrying overuse of these drugs in Iraqi healthcare. The results show most bacterial isolates were resistant to most antibiotics. S. aureus infection diseases have become more difficult because of the emergence of multidrug-resistant strains, especially MRSA, which has become a serious problem for healthcare professionals worldwide [18].

Many people develop MRSA, and some isolates have become resistant to other antibiotic types, like tetracyclines, aminoglycosides, macrolides, glycopeptides, and lincosamides [19]. This makes treatment harder. Aminoglycosides are good antibiotics that work against many types of staphylococcal infections. Beta-lactams and glycopeptides frequently combine with aminoglycosides to treat staphylococcal and enterococcal infections [20]. All S. aureus isolates showed a high sensitivity for linezolid and tigecycline. Linezolid and tigecycline are regarded as the most efficient antibiotics against MRSA strains due to limited antibiotics [21].

Cefoxitin induces the mecA gene more effectively than methicillin and oxacillin, which explains the increased sensitivity of the Kirby-Bauer disc diffusion technique according to guidelines (CLSI, 2020). Several studies have found that cefoxitin has more specificity and sensitivity than oxacillin [22].

The growth method on Congo red agar revealed an accurate way to distinguish the phenotypic pattern of slime layer-producing isolates with high virulence. It could also help differentiate between strong and weak slime layer production, which reflects the severity of the infection and aids in determining the initial treatment. The difference in the degree of production of the slime layer is due to the difference in the production of polysaccharide adhesion (PIA) and reflects the change in genetic regulation. The growth method was used on Congo red agar, an easy-to-use method, to investigate the production of the slime layer of several isolates. This method encourages the production of multiple polysaccharides in a rich medium, but it can be considered a minor specialty due to potential differences in the formation of the colonies' black pigment. Differences in the cultural medium used may affect the results of this method [23].

The results of the microtiter plate method revealed that all S. aureus isolates can produce biofilm; however, there could be many reasons for the differences in biofilm thickness, such as differences in the isolates' ability to make the biofilm, the main number of cells that stuck together, and the amount and quality of quorum-sensitive signaling molecules (auto-inducer) that each isolate made [24]. Biofilm production by S. aureus is a significant factor that contributes to chronic infections. It acts as an effective defense against the body's defenses and antibiotic therapy, making eradication extremely challenging [25].

According to the results of our study, ten isolates were negative for the icaD gene. However, phenotypically, the MTP method found these isolates to be biofilm formers. Two of them were weak biofilm formers, seven were intermediate biofilm formers, and one was a strong biofilm former. Several Staphylococcus species, including S. aureus, harbor the ica operon, which mediates polysaccharide intercellular adhesion (PIA). According to studies, deletion of the ica operon is due to a loss of the ability to produce PIA and form biofilms [26]. Biofilm formation is a significant advantage for S. aureus, allowing it to attach to surfaces and form a community resistant to antibiotics and host defenses. An important part of the S. aureus biofilm matrix is PIA, which is made of a protein that is controlled by the icaD gene [27]. The IcaD biofilm formation mechanism is complex, including the participation of many kinds of proteins, and so many genes are involved. The detection of biofilm-associated icaD gene was not well correlated with biofilm production on MTP methods. The presence of icaD gene-negative but biofilm-positive isolates can be accounted for in bap and ica-independent control of slime production/adhesion mechanisms [28-30].

CONCLUSION

Our study revealed that S. aureus is the predominant and main gram-positive bacteria in diabetic foot infections. The prevalence of S. aureus is significantly higher among males than females, particularly among patients with diabetes mellitus type 2 at ages 45 and 54 years old. Biofilm positivity was significantly higher for multidrug-resistant isolates of the tested antibiotics. However, the MTP method is an accurate and reproducible method for screening, and this technique can serve as a reliable quantitative tool for determining biofilm formation. All MRSA isolates form biofilms that make bacteria more resistant to antibiotics and the immune system, leading to chronic and hard-to-treat infections. Furthermore, when choosing empiric antibiotic therapy, one should take into account the high prevalence of Gram-positive S. aureus in DFIs and their resistance to beta-lactams, which emphasizes the need for continuous monitoring of the antibiotic resistance patterns of the frequently found isolates in chronic wound infections. Linezolid and tigecycline are useful treatment options for DFIs. Despite the isolation of a small amount of VRSA from DFIs, we do not recommend the empirical use of VRSA anti-biotherapy, as the common use of vancomycin to treat MRSA infections leads to the appearance of VRSA among MRSA isolates. The high frequency of the icaD gene

in MRSA isolates strengthens the association with biofilm formation in S. aureus isolates; it is considered an ica-dependent type. Consistently, mandatory caution is needed to control the spread of MRSA and VRSA. This finding provides a potential target for future diagnostic tests or treatment strategies.

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