ORIGINAL ARTICLE

Phenotypic and Genotypic Study of Biofilm Formation of Multi-Drug Resistant *Staphylococcus aureus* Isolated from Different Clinical Infections/Iraq

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ABSTRACT

Background*: Staphylococcus aureus is a main pathogen linked to nosocomial and community acquired infection and reason essential public health problem.* ***Objective:*** *The goal of this study was to assess the phenotypic and genotypic capacity for biofilm formation among MDR Staphylococcus aureus isolates from different clinical infections.* ***Methodology****: From November, 2024 to June, 2025, A total number of (230) specimens were collected randomly from patients with different clinical infections of different hospitals / Iraq. The microbiological assays were used to identify S.aureus isolate that exposed to antimicrobial resistance test towards 18 antibiotic types by using disc diffusion test and Biofilm formation detection of S. aureus isolates by using Micro Titer Plate with Congo Red Agar Method and PCR technique.* ***Results****: Out of 230 specimens, only (80) of S. aureus isolates were subjected to antimicrobial susceptibility test towards 18 antibiotic types, The results showed the highest rate of S. aureus resistance was Penicillin G (97.5%) and the low rate was Vancomycin (0%). Out of 80 specimens, 45(56.25%) isolates were MDR S. aureus and only 41(91.2%) isolates showed biofilm formation by CRA Method while 45 (100%) isolates were positive by Microtiter plate assay and using PCR technique to identify biofilm formation genes in 45 MDR S. aureus, IcaA gene were (88.8%) and the IcaD gene (88.8 %), whereas clfA gene (82.2%).* ***Conclusion****: This study concluded that MDR S. aureus isolates from patients with different clinical infections are capable of biofilm formation.*

INTRODUCTION

S. aureus is an essential pathogen that has a main effect on human health, infections caused by this pathogen circulated in community acquired as well as hospital-acquired settings, though it is a versatile human pathogen causing infections ranging from relatively mild involvement of skin and soft tissue to life-threatening sepsis, pneumonia and (TSS) toxic shock syndrome [1]. The pathogenicity of *S. aureus* is caused through a amount of traits, such as invasive components, virulence factors such as extracellular factors, toxins, adhesion, biofilm formation, and antibiotic resistance. These traits help these organisms in becoming Increasing resistant to hospital environments, increasing infections, and evading the immune system of the host [2].

Antibiotic resistance in *S.aureus* is connected by the presence of Mobile Genetic Elements, the main means by which genetic information is replaced among bacteria through horizontal gene transfer, which plays an important role in the capability of *S.aureus* to adapt to environmental stressors, such as antibiotics exposure [3]. Biofilm formation in *S. aureus* causes an increase in antibiotics resistance in chronic diseases, including osteomyelitis, endocarditis, and wound infection, the *S. aureus* biofilm structure is composed of polysaccharide, protein, and external DNA. Bacterial cells in the biofilm compose of resistant per sister cells and exhibit multidrug resistance and the emergence of multidrug resistant *S. aureus* strains is a health problem [4]. The biofilm-associated polysaccharide of *S. aureus* is known as the polysaccharide intercellular adhesion or (PIA). Consequently, biofilm formation is an essential stage in the pathogenesis of Staphylococci and relies on the expression of the *icaADBC* operon involved of the synthesis of this polysaccharide intercellular adhesion [5]. The current study aims to determine the association between genotypic and phenotypic biofilm formation in MDR *S. aureus* isolates.

METHODOLOGY

Samples collection

A total number of 230 samples were collected randomly from patients with different clinical infections referred to different hospitals in Al-Najaf province/Iraq including: Al-Sadir Medical City, AL-Zhraa Hospital, Al-Hakim General Hospital, Najaf General Hospital and the burn center in Najaf province, for different age

groups and for both gender during the period from (November, 2024 to June, 2025).

Ethical approval.

All participants supplied their informed agreement to be included in the study and were assured that their information would be kept private and used exclusively for this purpose.

Isolation and identification of *S. aureus*

All collected samples were transferred directly to the lab for isolation. Samples were inoculated into sterilized brain heart infusion broth and incubated at 37°C for 24 hours. Later on, samples were inoculated on mannitol salt agar and blood agar for overnight at 37ºC under aerobic condition for *S. aureus* diagnosis. For identification, biochemical test and Gram stain procedure was conducted [6]. A Vitek-2 compact system was employed for the confirmation of identification.

Antibiotics susceptibility testing

This research employed 18 types of commonly used antibiotic including Penicillin G 10 µg, Erythromycin 15 µg, Fusidic acid 10 µg, Tetracycline 30 µg, Chloramphenicol 10 µg, Azithromycin 15 µg, Cefoxitin 30 µg, Oxacillin 10 µg, Tobramycin 10µg, Ciprofloxacin 5 µg, Gentamicin 10 µg, Rifampicin 5 µg,Clindamycin 10 µg, Trimethoprim-sulfamethoxazole 1.25/23.7 µg, Amikacin 30 µg, Doxycycline 30 µg, Levofloxacin 5 µg and Vancomycin 30 µg.The antibiotic sensitivity phenotypes of bacterial isolates were conducted by the Kirby-Bauer disc diffusion assay on Mueller Hinton agar. Only 80 *S. aureus* isolates were subjected to antimicrobial susceptibility test towards 18 antibiotic types. The results were recorded in accordance with guidelines of CLSI [7].

Biofilm Formation

MicroTiter Plate (MTP) method

Biofilm detection of *S. aureus* isolates was carried out according to [8], Bacteria were streaked into BHI medium supplemented with 1% glucose from blood agar plates and grown at 37° C. After the turbidity of bacterial stock arrives to 0.5 McFarland standard, 200 µl of bacterial solution was vaccinated into sterile MTP wells excepting the wells of the latter segment that was indicated as a negative control containing sterile MHP. The vaccinated plates were incubated for 48 h. The items in the wells were emptied, and the residues of microbes in the wells were washed with phosphate buffered saline (PBS) to eliminate planktonic cells. The plates were subsequently dried for 1hrs at 60°C prior to staining with 150 µl of crystal violet (0.1%) for 15 min at room temperature and washed with PBS. The microtiter plate were then reversed and employed vigorously on filter paper to eliminate any excess liquid. The microtiter plates were air dried. Biofilm formation was measured by solubilizing the CV stain with 150 µl of 95% ethanol. An ELISA reader interpreted the outcomes at 630 nm. Deka's measures finished understanding and reviewing (non-low-moderate-strong) biofilm creation. The optical density (ODT) of each isolate was gained by the arithmetic mean of the absorbance of three wells, and this value was equated with the absorbance of negative controls (ODc).

Congo Red Agar Method

Congo red agar is a specifically formulated medium consisting of brain heart infusion (BHI) broth (37 g/l), added with sucrose (50 g/l), agar No1 (10 g/l), and Congo red (0.8 g/l). A concentrated aqueous solution of the Congo red stain was prepared and subsequently autoclaved at 121ºC for a duration of 15 minutes. Subsequently, this solution was introduced to the autoclaved BHI agar along with sucrose at a temperature of 55ºC. The CRA plates prepared at that time subjected to inoculation with *S. aureus* isolates and were aerobically incubated at a temperature of 37°C for a period of 24 hours. The presence of dry crystalline colonies on the CRA plates was indicative of biofilm formation, while the colonies of non-biofilm producers keep on either pink or red in color. This method was employed for ensuring sterility and for differentiating non-specific binding [9].

Molecular Detection of *S. aureus* genes by conventional PCR technique

The polymerase chain reaction evaluate was performed for the purpose of confirmatory diagnosis and to investigate some resistance genes of *S. aureus* isolates. The examination consists of the following steps:

Bacterial Genomic DNA Extraction

The Genomic DNA Extraction Kit from (Magen/china) was utilized to extract DNA from (45/80) MDR S. aureus isolates. The concentration of the DNA was evaluated through spectrophotometric analysis, which involved evaluating its optical density at 260 nm (the Extinction coefficient of dsDNA being 50 µg/ml at 260 nm). The purity of the DNA solution can be ascertained by the ratio of OD 260-280 falling within the range of 1.8±0.2 for pure DNA. The thermocycler employs a specific PCR program. Subsequently, the PCR products and the ladder marker are separated through electrophoresis on a 1.2% agarose gel [10].

Polymerase Chain Reaction (PCR) Technique

In the present investigation, monoplex PCR methodology was utilized for the identification of various genes responsible for encoding virulence factors and antibiotics resistance within isolates of MDR S. aureus isolates. The application of monoplex PCR was specifically for the purpose of detection *IcaA, IcaD* and *clfA* gene responsible for biofilm formation. The PCR mixture was prepared with a total volume of 20 µl, comprising 4 µL of Master mix, along with 2µl of each primer, Nuclease -free water 9.8 µl and 5µl of extracted DNA, followed by vertexing. The negative control included all components but except the template DNA, which was substituted with distilled water. Subsequently, the PCR reaction tubes were briefly

centrifuged to ensure thorough mixing and settling of contents at the tube bottom before being transferred to a thermocycler PCR instrument for DNA amplification. as detailed in (Tables 1).

PCR Thermo - cycling conditions

The PCR tubes were located on the PCR machine and the right PCR cycling program parameters environments were installed as in (Table 2).

Table 1: The primer used in this study

Primer Type	Primer sequence (5'- 3')	Product Size (bp)	Reference
IcaA	**F** 5'- TCTCTTGCAGGAGCAATCAA-3' **R** 5'- TCAGGCACTAACATCCAGCA-3'	**188 bp**	[11]
IcaD	**F** 5'- ATGGTCAAGCCCAGACAGAG-3' **R** 5'-AGTATTTTCAATGTTTAAAGCAA -3'	**198 bp**	[11]
clfA	**F** 5'- ATTGGCGTGGCTTCAGTGCT -3' **R** 5'- CGTTTCTTCCGTAGTTGCATTTG -3'	**288bp**	[12]

Table 2: PCR thermo-cycling conditions of genes amplification used in this study

Genes name	Steps	Temperature (ºC)/ Time	No. of cycle
IcaD,IcaA	Initial denaturation	94ºC/5min	1
	DNA denaturation	94 ºC \30 sec.	50
	Primer annealing	55.5 ºC \30 sec.	
	Extension	72 ºC \30 sec.	
	Final extension	72 ºC \1min	1
Clf A	Initial denaturation	94ºC/5min	1
	DNA denaturation	94ºC/60sec	25
	Primer annealing	55 ºC \60 sec.	
	Extension	72ºC/60sec	
	Final extension	72 ºC \10min	1

Finally, each amplified PCR products were subjected to agarose gel electrophoresis and the specific size of PCR products was assessed by comparing with the ladder bench top PCR markers.

RESULTS

Isolation and Identification of *S. aureus*

A total number of (230) patients submitted in this study who suffered from different clinical infections. The samples showed bacterial only 195 (84.78%) specimens gave bacteria growth, while 35 (15.21%) specimens did not show "bacteria" growth. Only 80/195(84.21%) isolates were recognized as *S. aureus*.

Antibiotic susceptibility test of *S. aureus* isolates

In this study, only (80) of *S. aureus* isolates were subjected to antimicrobial susceptibility test towards 18 antibiotic types. The results of antibiotics resistance test of *S. aureus* isolated from different clinical infections were shown as in (Table 3).

In the present study, three classes of cell wall synthesis inhibitors were tested contain four types of antibiotics include (PEN, FOX, OXA and VAN). The results showed the highest rates of resistance towards the antibiotic Penicillin G with a percentage of 78(97.5%) followed by Cefoxitin (57.5%) and Oxacillin (56.25%) and no resistant to Vancomycin (0%).

Biofilm formation for *S. aureus*

Congo Red Agar Method (CRA)

45 (100 %) of *S. aureus* isolates were multidrug resistant by using CRA Method, 41(91.2%) of *S. aureus* isolates showed dry crystalline colonies on the CRA plates while 4 (8.8%) did not produce biofilm (Figure 1).

Table 3: Antibiotics resistance percentage of (80) *S. aureus* isolated from different clinical infections

Name of Antibiotic		Diameter of Inhibition Zone (mm)		
		Sensitive (S)%	Intermediate (I)%	Resistance (R) %
β-lactams / Penicillins (penams)	Penicillin G (PEN)	2(2.5%)	0	78(97.5%)
	Oxacillin (OXA)	32(40%)	0	48(60%)
β-lactams / Cephems	Cefoxitin (FOX)	30(37.5%)	0	50 (62.5%)
Glycopeptides	Vancomycin (VAN)	78(97.5%)	2(2.5%)	0
Aminoglycoside	Gentamicin (GEN)	32(40%)	5 (6.25%)	43(53.75%)
	Amikacin (AK)	41(51.25%)	4(5%)	30(43.75%)
	Tobramycin (TOB)	37(46.25%)	3(3.75%)	40 (50%)
Macrolides	Azithromycin (AZM)	29 (36.25%)	2(2.5%)	49(61.25%)
	Erythromycin (Ery)	17(21.25%)	4(5%)	59(73.75%)
Tetracyclines	Tetracycline (TET)	24(30%)	4(5%)	52(65%)
	Doxycycline (DXT)	42(52.5%)	6(7.5%)	32(40%)
Ansamycins	Rifampicin	42 (52.5%)	2(2.5%)	36 (45%)
Lincosamides	Clindamycin (CLI)	41(51.25%)	3(3.75%)	36 (45%)
Quinolones / Fluoroquinolones	Ciprofloxacin (CIP)	34(42.5%)	2(2.5%)	44(55%)
	Levofloxacin (LEV)	59(73.75%)	1(1.25%)	20(25%)
Sulfonamides	Trimethoprim-sulfamethoxazole (SXT)	42(52.5%)	3(3.75%)	35(43.75%)
Fusidance	Fusidic acid (FUS)	19(23.75%)	4(5%)	57(71.25%)
Phenicols class	Chloramphenicol (C)	24 (30%)	5(6.25%)	51(63.75%)

Fig. 1: Biofilm formation by some *S. aureus* isolates

Table 4: Biofilm production by micro titer plate method for *S. aureus* isolates

Type Biofilm	NO. (%) of *S. aureus*
Strong (> 0.240 ± 0.022)	25 (55.5%)
Moderate (0.120-0.240 ± 0.020)	14 (31.2%)
Weak (< 0.12 0 ± 0.012)	6 (13.3%)
Total	**45(100%)**

Microtiter plate assay (MPA)

The results showed the capacity of 45 MDR *S. aureus* isolates to biofilm formation, that most resistant to antibiotics (multi drug resist antibiotic) by using The microtiter plate assay (MPA), from the more resistance 45 (100%) isolates of *S. aureus,* 25/45(55.5%) were strong biofilm producers, 14/45(31.2%) were moderate and 6/45(13.3%) were weak comparison with control as shown in (Table 4) and (Fig 2).

Molecular detection of Biofilm formation of MDR *S. aureus* isolates

Biofilm-encoding genes were detected using conventional PCR; the presence of the *IcaA*, *IcaD* and *clfA* genes was examined by PCR technique. Out of 45 MDR *S. aureus* isolates; 40(88.8 %) were positive for *IcaA* gene, also the *IcaD* gene was detected in *S. aureus* which was in 40 (88.8 %) isolates, while 37 (82.2%) were positive for *clfA* gene as shown in (Figures 3,4,5).

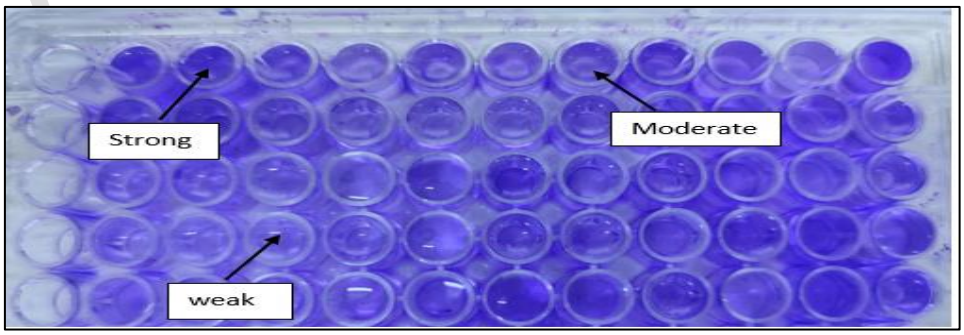

Fig. 2: Biofilm formation of *S. aureus* by (MPA) method

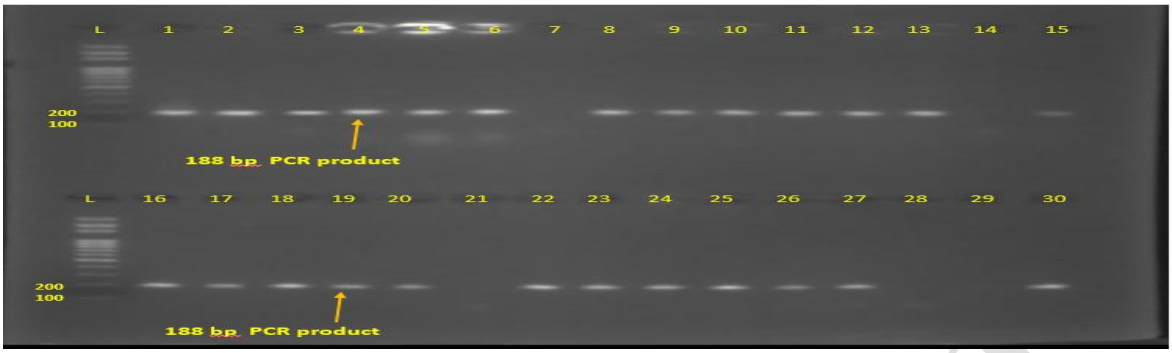

Fig. 3: Agarose gel electrophoresis with ethidium bromide stained of mono-plex PCR amplified product from extract DNA of *S. aureus* isolates with (*IcaA*) gene (amplified size 188bp)

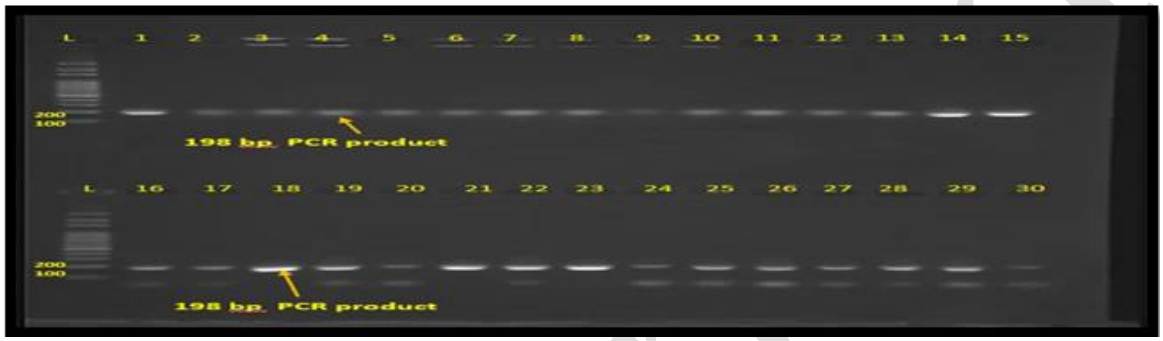

Fig. 4: Agarose gel electrophoresis with ethidium bromide stained of mono-plex PCR amplified product from extract DNA of *S. aureus* isolates with (*IcaD)* gene (amplified size 198bp)

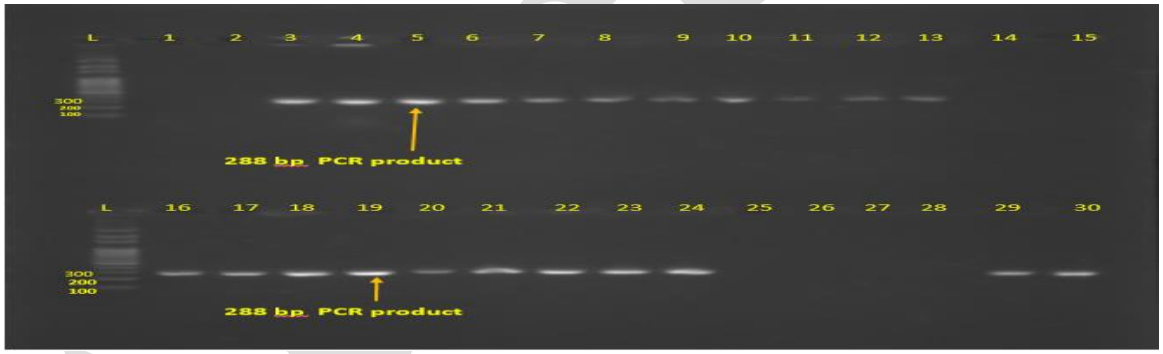

Fig. 5: Agarose gel electrophoresis with ethidium bromide stained of mono-plex PCR amplified product from extract DNA of S. aureus isolates with (clfA) gene (amplified size 288bp).

DISCUSSION

S. aureus is an important human pathogen that has a major impact on public health. Nearly 50% of the human population are asymptomatic carriers of *S. aureus*, However, some groups of people are at higher risk of *S. aureus* colonization (up to 80%), including health care workers, diabetic persons, patients on intravenous drug individuals with weak immunity [١٣].Beta-lactamase is an enzyme synthesized by various bacterial species that confers resistance to β-lactam antibiotics, including penicillin, cephamycin, and carbapenem, β-lactamase causes antibiotic resistance by damaging the structure of the antibiotic, and The β-lactamase enzyme opens the β-lactam ring as a result of hydrolysis., eliminating the antibacterial characteristics of the molecule[14]. The first mechanism of resistance in *S. aureus* is the expression of β-lactamase enzyme are encoded by the *blaZ* gene that hydrolyzes the β-lactam ring and so creating the antibiotic inactive and second, the specific acquirement of staphylococcal *SCCmec* (Cassette chromosome mec), which carry *mecA* gene that encodes for a protein called penicillin binding protein (PBP2a) [15]. Other studies by Abd Al-Mayahi [16], who found all isolates was resistant to Penicillin, cefoxitin (100%) and (77.8%) resistance rate for Oxacillin. Also, Abd Zaid were reported the highest level of resistance was with Penicillin, Cefoxitin 100% and Oxacillin 81.92% [17]. In the current study showed

the percentage of resistance to Aminoglycoside, like Gentamicin (53.75%), Amikacin) 43.75%)and Tobramycin (50%) and *S. aureus* was most resistant to Macrolide antibiotics, like Erythromycin (73.75%) and Azithromycin (61.25%) and also resistant to Ciprofloxacin, Levofloxacin, Trimethoprim-sulfamethoxazole and Rifampicin were 55%, 25%, 43.75% and 45%, respectively. *S. aureus* resist to Fluoroquinolones via the modification of the FQ targets that results from mutations in genes coding for DNA gyrase and topoisomerase IV, overexpression of efflux pumps, plasmid-mediated resistance, and due to decrease drug accumulation[18]. The percentage of resistance in this study to fusidic acid was (71.25%) and Chloramphenicol was (63.75%) was identified by other studies[19, 20], they reported higher rate of resistant isolates to fusidic acid was (71.25%, 86.7%) respectively, while, Aziz reported rate of isolates resistant to Chloramphenicol was (45%) [21].

The capacity of a microorganism to create biofilm is an essential virulence factor. These biofilms are the main responsible for many chronic infections such as diabetic foot ulcers, and they create the way for the reemergence of multidrug-resistant strains and result in therapeutic failure[22].

Bacteria produce three-dimensional structures called biofilms that adhere to surfaces covered in a self-produced extracellular matrix, this matrix not only act as physical barrier against the host immune system and antibiotics, but also facilitates the persistence and endurance of bacteria within the biofilm environment [23].

In *S. aureus* biofilms, the primary component of the EPS is the polysaccharide intercellular adhesin (PIA), which is encoded by the ica operon (*icaABCD*), PIA possesses a positive charge and plays important roles in bacterial colonization, biofilm formation, and biofilm-associated infections. It as well helps to immune evasion, resistance to antimicrobials and defense against phagocytosis [24].

Moreover, *S. aureus* produce a family of adhesion molecules called as microbial surface components, that know adhesion matrix molecules (MSCRAMM), these molecules are encoded by several genes, including fnbA and fnbB (fibronectin-binding protein A/B), *clfA* and *clfB* (clumping factor A/B), and *fib* (fibrinogen-binding protein), These proteins encoded by these genes play an essential role in adhesion of host cells and pathogen recognition along with extracellular matrix proteins, which are the primary stages in biofilm formation and disease development [25].

Other results reported they presented the common biofilm-encoding genes were *icaA* (76.7 %) [26] and also *icaD* (70 %) while *clfA* (65.0 %) of *S. aureus* isolates, also, Chan [27], who showed that the presence of also clumping factors A and B (*clfA and clfB*) genes and intracellular adhesion A and D (*icaA and icaD*) gene was (100 %) in all *S. aureus* isolates.

CONCLUSION

The present study has shown a great emergence of MDR *S. aureus* isolates from patients for different clinical infections and MDR *S. aureus* resistance for most commonly used antibiotics. The highest rate of resistance was seen with a a pencillin G and the low rate resistance was seen with vancomycin and also the ability of some MDR *S. aureus* isolates to produce biofilm. The study showed high percentage of MDR *S. aureus* have *IcaA*, *IcaD* and *clfA* genes responsible for biofilms formation.

Authors' contributions: All authors have contributed in the design, analysis, and interpretation of data, drafting and revising of the manuscript, and they have approved the manuscript as submitted**.**

Availability of data: All data are included in the manuscript and any other data are available upon reasonable request.

Conflict of interest: This study has not been published before and is not under consideration in any other reviewed media. All authors report no conflict of interest relevant to this work.

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