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Biofilms formation and relationship to gene-producing biofilms in Staphylococcus aureus Isolated from clinical specimens

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ABSTRACT

Staphylococcus aureus found on human skin, in the nose, armpit, groin, and other areas. While these germs don't always cause harm, they can make you sick under the right circumstances. *S. aureus* is the leading cause of skin and soft tissue infections, such as abscesses, boils, furuncles, and cellulitis (red, swollen, painful, warm skin). *S. aureus can* also cause more serious infections, such as pneumonia, bloodstream infections, endocarditis (infection of the inner lining of the heart chambers and heart valves), and bone and joint infections. The present study evaluated biofilm forming capacity and the presence of biofilm related genes (*icaA, icaB, icaC, icaD, cna, atl, fnbA* and *fnbB*) among *S. aureus* strains isolated from patients undergoing ureteral catheterization.

Different clinical specimens were collected from 253 patients. 137 isolates of *S. aureus* were identified by conventional microbiological methods, and isolates were tested for their capability to form biofilm using the microtiter plate assay (MTP) and Congo red agar (CRA). The existence of biofilm associated genes was determined by molecular technique. Relations among those methods were as a final point evaluated by statistical analysis.

One hundred and thirty-seven isolates of *S. aureus* were isolated and identified from 253 clinical specimens (54.15%). Out of the positive isolates, 100% were biofilm producers with MTP assay and 89% with CRA method. *S. aureus* isolates were further classified as high (21%), moderate (71%), weak (8%) and non–biofilm producers (0). All biofilm producing strains were positive for *icaC, icaD* and *cna* genes, except SA88 isolate which negative for these genes. There was a significant difference between MTP assay with CRA method for biofilm formation ($p<0.0001$). Ninety six percent of biofilm forming *S. aureus* were possess *icaC, icaD* and *cna* genes, which indicate the important role of *ica* genes as virulence markers in *S. aureus* infections associated with urinary tract catheterization.

Keywords: *S. aureus; Biofilm genes; PCR technique.*

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INTRODUCTION

Staphylococcus aureus is a leading cause of skin structure infections particularly associated with urinary tract catheters [1]. *S. aureus* produces a number of virulence factors that provide the ability to colonize for it, adhere to surfaces and form biofilm, invade or evade the immune system, develop resistance to multiple antibiotics and cause toxicity to the host [2][3]. *S. aureus* is commonly observed colonizing several parts of the body in healthy individuals [4], and causing of biofilm associated infections [5] such as catheter related sepsis. The ability of *S. aureus* to form an extracellular slime and constitutive a biofilm assists this organism to endure the host immune response and to make clinical treatment awfully difficult because of biofilm formation defends bacteria from antimicrobial agent [6].

Biofilms are aggregations of microbial cells that are usually adhere to a surface and are held together by an extracellular matrix [7]. Matrix constituents are frequently created by the cells that make up the biofilm and work as natural glue, linking neighboring cells to one another [8]. Biofilms have been shown to contribute to the recalcitrance of infections, in part due to enhanced antibiotic tolerance conferred by the matrix itself, which can decrease permeation of antimicrobial agents [8][9]. Generally, biofilm expansion occurs through a mechanism termed 'quorum sensing' in which the expression of specific signal molecules, called 'autoinducers', is regulated in response to cell density and stressed environmental conditions [10].

Two key factors have been linked with suboptimal outcomes in treating such invasive *S. aureus* infections: (i) the bacteria's capacities to grow resistance to multiple antibiotics (e.g., methicillin resistant *S. aureus* (MRSA), Vancomycin intermediate *S. aureus* (VISA), and Vancomycin resistant *S. aureus* (VRSA) [11], and (ii) its ability to form biofilms on each instinctive tissues and implanted biomaterials [5]. It is well known that *S. aureus* cells within a complex biofilm matrix are refractory to both systemic antimicrobial agents and host immune responses [12][13][14]. *S. aureus* is able to form a diverse multilayered biofilm using different schemes, including: expression of numerous surface proteins; (ii) release of extracellular DNA; and expression of the polysaccharide intercellular adhesin (PIA) by the *icaADBC* operon [10].

Antibiotic resistance seems to be joined to biofilm formation, with methicillin sensitive *S. aureus* (MSSA) strains more likely to necessitate the polysaccharide intercellular adhesin (PIA), whose production is ruled by the *ica* operon, whereas methicillin–resistant *S. aureus* (MRSA) strains most often create biofilms in a PIA-independent, glucose–dependent manner [15]. The intracellular adhesion (*ica*) cluster, *icaADB* and *C*, encodes enzymes mediating cell–cell the adhesion and biosynthesis of the polysaccharide intercellular adhesion (PIA) which is vital for biofilm formation [16] [17] [18] [9]. Another feature of *S. aureus* which assists the pathogen's escape from protective immune responses express a number of immune-modulating proteins [19]. Staphylococcal complement inhibitor (SCIN) is one of the immune-modulating proteins, a complement inhibitor, hindering the ability of human neutrophils to opsonophagocytose of *S. aureus* and neutrophil chemotaxis [20][21]. This study aims to estimate the resistance of S. *aureus* toward drug, biofilm formation and prevalence of biofilm forming genes in *S. aureus* collected from two major hospitals in Erbil city and determines the relationship between MRSA, β–lactamase, *mecA* gene, phenotypic biofilm formation and biofilm related genes.

MATERIALS AND METHODS

Clinical isolate repository

The study was conducted in Department of Biology, Salahaddin University-Erbil, Iraq. The ethical approval was obtained from institutional review board of Salahaddin University council. A total of 253 samples (98 urine, 65 wound swab, 39 burn, 27 tonsils, stool and 24 sputum) were taken for the study. The samples were collected in clean, sterile and leak proof containers using aseptic technique by experienced medical officers and taken immediately into the microbiology laboratory for further processing. All the samples were inoculated in brain heart infusion broth then incubated for 24 hrs. with shaking at 250 rpm, after that, the positive growth cultured Blood Agar (BA), MacConkey agar (MA) and Mannitol Salt Agar (MSA) and incubated at 37°C for 24 hrs. The isolates were identified as *S. aureus* using standard microbiological techniques including biochemical, slide and tube coagulase tests.

Culture and identification

The assumed colonies of *S. aureus* were identified by using conventional methods; include gram staining, biochemical tests which comprise: catalase, coagulase using tube coagulase test (TCT), and DNase with methyl green. The final methods used for identify the *S. aureus* was polymerase chain reaction (PCR) method built on 16S rRNA and *nuc* genes was used. The sequences of both genes and PCR program were mentioned in Table 2.

Antimicrobial susceptibility screening

Antimicrobial sensitivity testing was performed by disk diffusion method according to the Clinical and Laboratory Standards Institute references (CLSI) [22], toward the following antimicrobials; Amikacin 30 µg, Azithromycin 15 µg, Ciprofloxacin 5 µg, Clindamycin 2 µg, Erythromycin 15 µg, Gentamicin 10 µg, Levofloxacin 5 µg, Netilmicin 30 µg, Nitrofurantoin 300 µg, Norfloxacin 10 µg, Oxacillin 1 µg, Penicillin 10 U, Tetracycline 30 µg, Tobramycin 10 µg, Trimethoprim+Sulfamethoxazole 1.25+23.75 µg, and Vancomycin 30 µg. A lawn of test *S. aureus* was prepared by evenly spreading 100 µl inoculums ($1.5*10^8$ CFU/ml) according to 0.5 McFarland standard solution with the sterilized swab on top of the entire surface of Mueller Hinton Agar plate. The disks were resolutely applied onto the agar plates surface within 15 minutes of inoculation [9].

Assessment of biofilm synthesizing S. aureus

Congo red agar method

Phenotypic production of biofilm in all *S. aureus* isolates was assessed by culture on CRA plates explained by [23] [24]. First, CRA plates were prepared by addition of 0.8 g Congo red (Merck, Germany) and 36 g of sucrose (Sigma, USA) to one liter of brain heart infusion agar (BHI agar, Merck, Germany). The plates were incubated at 37 ºC for 24 hrs. The morphology of colonies was then interpreted based on colony color as Bordeaux pink (red), almost black, black, and strong black. Strains with red colonies were classified as strains unable to produce biofilm, while almost black colors were indicative of a weak biofilm production activity. Conversely, very black and black colonies were considered as strong biofilm producer strains.

Polystyrene microtiter plate assay

Biofilm production quantitatively was determined through a modified Microtiter plate method as defined by [25]. Briefly, bacterial isolates were grown in trypticase soy broth with 0.5% glucose and incubated overnight at 37 ºC. Dilute the cultures with 1:40 in fresh TSB with 0.5% glucose. Then added 200 µl of the diluted solution in to Microtiter plate wells and incubated at 37 ºC for 48 hrs. The negative control wells contained only 200 µl of TSB–0.5% glucose without bacterial suspension. Gently wells washed 3 times with phosphate buffered saline (PBS; pH 7.2), and fixed using methanol for 20 min, leaved at room temperature for drying, and then stained with 0.1% crystal violet. The dye bound to the adherent cells was dissolved with 1 ml of 95% ethanol per well. As a final point, the optical density (OD) at 570 nm of each well was measured using ELISA reader. Calculated the average OD of negative control + 3 × standard deviation (SD) of negative control which described as Optical density cut–off (ODc). Based on the absorbance of the crystal violet-stained attached cells, analyzed and categorized of biofilm was formed by strains (Table 1).

TABLE 1: Classification of biofilm formation abilities by Microtiter plate method

Cut-off value calculation	Mean of OD570 values results	Biofilm formation abilities
OD > 4×ODc	OD > 0.557	Strong
2×ODc < OD ≤4×ODc	0.278< OD ≤ 0.557	Moderate
ODc< OD ≤ 2×ODc	0.139< OD ≤ 0.278	Weak
OD ≤ 0.139	OD ≤ 0.139	None

Genomic DNA extraction

Genomic DNA was extracted from pure cultures *via* the Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan) according to the fitting protocols in the manufacturer's instructions; through a 100 µl as a final elution amount.

Extracted DNA was stored at –20°C until necessary for PCR. For evaluating the concentration and purity of DNA, the NanoDrop 1000 spectrophotometer was used in which 1 µl of the DNA genome was used for define the concentration and purity of DNA samples.

TABLE 2. Sequences of oligonucleotide primers used for PCR amplification of genes-formation biofilm with 16S rRNA and *nuc* genes used in this study

Functional category	Primers detail			References
	Primer Sequence (5′ – 3′) (Oligonucleotide)	**Amplicon size (bp)**	**Cycling program**	
16S rRNA	CACCTTCCGATACGGCTACC GTTGACTGCCGGTGACAAAC	372	95°C–30 s; 59°C–45 s; 72°C–1 min; 35 cycles	(Gowrishankar et al., 2016)
nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAG C	279	95°C–30 s; 53°C–45 s; 72°C–40 s; 40 cycles	(Blaiotta et al., 2004)
icaA	ACA CTT GCT GGC GCA GTC AA TCT GGA ACC AAC ATC CAA CA	188	94°C–30 s; 56°C–60 s; 72°C–45 s; 30 cycles	(Kouidhi et al., 2010)
icaB	CCC AAC GCT AAA ATC ATC GC ATT GGA GTT CGG AGT GAC TGC	1080	95°C–30 s; 58°C–30 s; 72°C–45 s; 40 cycles	(Gowrishankar et al., 2016)
icaC	CTT GGG TAT TTG CAC GCA TT GCA ATA TCA TGC CGA CAC CT	209	95°C–30 s; 55°C–40 s; 72°C–45 s; 40 cycles	(Nourbakhsh and Namvar, 2016)
icaD	ATG GTC AAG CCC AGA CAG AG CGT GTT TTC AAC ATT TAA TGC AA	198	94°C–30 s; 55°C–40 s; 72°C–45 s; 30 cycles	(Kouidhi et al., 2010)
cna	CGA TAA CAT CTG GGA ATA AA ATA GTC TCC ACT AGG CAA CG	716	95°C–30 s; 54°C–40 s; 72°C–45 s; 35 cycles	(Tang et al., 2013a)
atl	GCC TGT TGC AAA GTC AAC AA CAC CGA CAC CCC AAG ATA AG	600	95°C–30 s; 56°C–30 s; 72°C–45 s; 40 cycles	(Gowrishankar et al., 2016)
fnbA	GAT ACA AAC CCA GGT GGT GG TGT GCT TGA CCA TGC TCT TC	191	95°C–30 s; 57°C–1 min; 72°C–1 min; 35 cycles	(Kouidhi et al., 2010)
fnbB	GAC CTG CTT CGC TAT CCA CA AGT CGT AAT GGC GAC AGG TG	980	95°C–30 s; 57°C–30 s; 72°C–1 min; 40 cycles	(Gowrishankar et al., 2016)

PCR screening of the gene-biofilm formation

Screening for biofilm related genes in *S. aureus* isolates was performed, using PCR technique. PCR reaction was conducted on the final volume of 25 µl using 12.5 µL of 2x Master mix, 1 µL of the DNA template, 1 µL of each primer (10 pmol) and 9.5 µL of nuclease-free water. DNA amplification was done in a thermocycler. Primers and amplification conditions for PCR programs were mentioned in Table (2), and the amplified products were subjected electrophoresis using 1.2% agarose gel containing 1x safe dye.

DATA ANALYSIS

Statistical Package for the Social Science Software (SPSS 26.0) (SPSS Inc., USA) used for statistical analysis. Statistical significance was assessed *via* the *chi-square* test, the test was used to analyze association between methods for detection of biofilm formation and biofilm related genes, and *p-value* <0.05 was considered statistically significant.

RESULTS

Isolation and characterization of S. aureus

From total screened clinical specimens, 137 (54.15%) were identified as *S. aureus* based on biochemical tests (Table 3). All isolates of *S.*

aureus were evaluated for the presence of the 16S rRNA and *nuc* genes for characterization and confirmation up to the species level. Based on PCR investigation, all the strains were confirmed as *S. aureus* by the occurrence of 16S rRNA and *nuc* genes (Figure 1).

TABLE 3. Identification of *S. aureus* isolates depending of morphological, cultural, biochemical tests, VITEK II system and molecular study

Biochemical tests	% Positive results
Coagulase	137
Catalase	137
Hemolysis	98 β, 3 α, 36 γ
DNase	137
16S rRNA gene	137
nuc gene	137

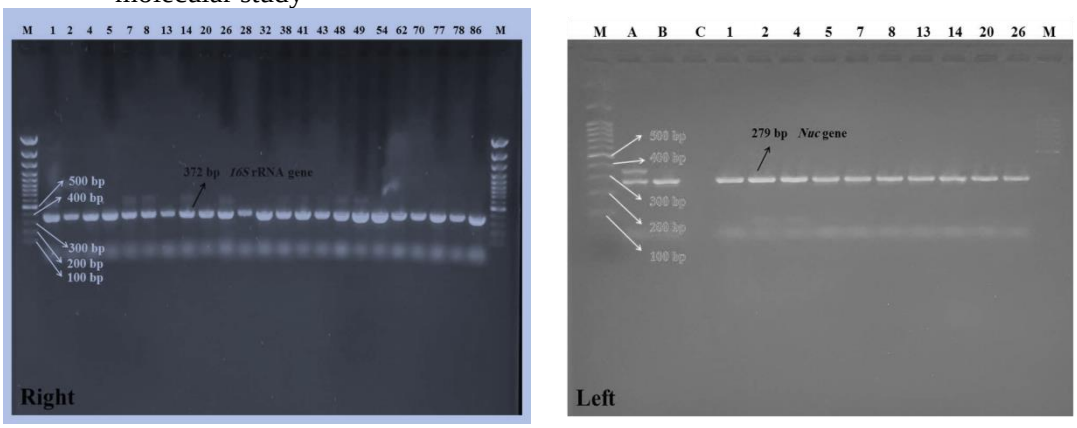

FIGURE 1. Agarose gel electrophoresis of PCR amplification products of *S. aureus*. *Right*: 16S rRNA gene, M: The DNA marker (100 bp ladder), lanes (1–86) positive amplification of 372 bp for *16S* rRNA gene. *Left*: *nuc* gene, M: The DNA marker (100 bp ladder), lane A and B: *S. aureus* ATCC23925 and ATCC43330 respectively, lane C: *S. epidermidis* ATCC22922, lanes (1–26) positive amplification of 279 bp for *nuc* gene.

Susceptibility pattern of S. aureus

The results antimicrobial sensitivity test for all *S. aureus* isolates toward 16 antimicrobials showed variable sensitivity patterns (Table 4). The highly resistant percentage was seen toward oxacillin (99.27%) followed by penicillin (97.08%) and the lowest resistant 1.46% was toward each of gentamycin, nitrofurantoin and trimethoprim-sulfamethoxazole.

TABLE 4. Susceptibility patterns of *S. aureus* isolate toward antimicrobials

Antimicrobials	Resistance *n.* (%)	Intermediate *n.* (%)	Sensitivity *n.* (%)
AK	2 (1.46)	2 (1.46)	133 (97.08)
AZM	64 (46.72)	5 (3.65)	68 (49.64)
CIP	15 (10.95)	2 (1.46)	120 (87.59)
CD	41 (29.93)	26 (18.98)	70 (51.09)
E	73 (53.28)	8 (5.84)	56 (40.88)
G	2 (1.46)	5 (3.65)	130 (94.89)
LEV	12 (8.76)	0 (0.00)	125 (91.24)
NET	2 (1.46)	4 (2.92)	131 (95.62)
NIT	29 (21.17)	2 (1.46)	106 (77.37)
NOR	25 (18.25)	47 (34.31)	65 (47.45)
OX	136 (99.27)	0 (0.00)	1 (0.73)
P	133 (97.08)	0 (0.00)	4 (2.92)
TE	32 (23.36)	4 (2.92)	101 (73.72)
TOB	26 (18.98)	4 (2.92)	107 (78.10)

SXT	2 (1.46)	11 (8.03)	124 (90.51)
VA	52 (37.96)	34 (24.82)	51 (37.23)

Biofilm formation through microtiter plate test

The ability to form a biofilm was evaluated using the MPM according to a previously described protocol [25]. In this study, OD_{570} mean of microplate readings after crystal violet staining ranged from 0.216 to 0.827. The mean of negative control was 0.054. An ODc_{570} of biofilm formation was defined as 0.139. The strains were divided into four groups: non–biofilm producer (−), $OD_{570} \leq 0.139$; weak biofilm producer (+), $0.139 < OD_{570} \leq 0.278$; moderate biofilm producer (++), $0.278 < OD_{570} \leq 0.557$; strong biofilm producer (+++), $0.557 \leq OD_{570}$. Our data showed that 100% (100/100) of the total *S. aureus* were biofilm positive. Furthermore, over the 100 *S. aureus* isolates, 21% showed biofilm forming ability with strong (*n= 21*), 71% moderate (*n= 71*) and 8% weak (*n= 8*) levels.

Biofilm formation determination by Congo red agar test

In vitro biofilm formation by CRA method differ from with MTP assay and the results shown that 48.9% of the isolates showed strong biofilm formation (strong black), 15.33% were moderate biofilm producer, 24.82% weak biofilm former and 11% of isolates were classified as non–biofilm producer. So, there were a significant different ($p<0.0001$) among both MTP assay and CRA method for detection of phenotypic biofilm formation among isolates of *S. aureus* (Table 7).

TABLE 7. Screening of *S. aureus* isolates from biofilm production by CRA and MPM assay.

Biofilm formation status	Screening method		*P* value
	CRA *n.* (%)	MTP *n.* (%)	
Strong	67 (48.90%)	29 (21.17%)	<0.0001
Moderate	21 (15.33%)	97 (70.8%)	
Weak	34 (24.82%)	11 (8.03%)	
None	15 (10.95%)	0 (0.00%)	

Detection of genes involved in biofilm formation

As the prime intention of the present study is the genotypic characterization of biofilm responsible genes, PCR assay was employed to detect *icaA*, *icaB*, *icaC*, *icaD*, *cna*, *atl*, *fnbA* and *fnbB* genes among 25 isolates of *S. aureus*. We evaluated the relationship between biofilm formation and the 8 predominant biofilm related genes. The distribution of these genes in 25 *S. aureus* isolates are shortened in Table 8. All the eight genes were detected among isolates with different frequencies. As can be seen, the majority of *S. aureus* isolates (96% [*n=24*]) were found to be positive for *icaC*, *icaD* and *cna* gene. The prevalence rates of the *icaA*, *icaB*, *atl*, *fnbA* and *fnbB* genes were unswervingly found to be 80, 72, 80, 86, and 60% respectively. In 32% (*n=8*) of isolates all of the studied genes were detected and in these isolates were biofilm producer. In the present study, there were significant association between presence of *icaA* ($p = 0.003$), *icaB* ($p = 0.028$), *icaC* ($p< 0.0001$), *icaD* ($p< 0.0001$), *cna* ($p< 0.0001$), *atl* ($p = 0.003$) and *fnbA* ($p< 0.0001$) genes and phenotypic biofilm formation in *S. aureus* isolates, while this association was not observed only for *fnbB* ($p = 0.317$).

TABLE 8. Relationships among the biofilm related genes and biofilm formation (MTP assay and CRA method) in *S. aureus* isolates.

Strain	*In vitro* Adherence (MTP) Assay		Biofilm phenotype on CRA	Biofilm producer	Presence of biofilm related genes							
	Adherence A_{570} nm Mean	Adherence Ability			*icaA*	*icaB*	*icaC*	*icaD*	*cna*	*atl*	*fnbA*	*fnbB*
SA01	0.236	+	Black*	Producer	+	+	+	+	+	+	—	+
SA02	0.488	++	Strong	Producer	+	—	+	+	+	+	+	+

			Black									
SA04	0.608	+++	Strong Black	Producer	+	+	+	+	+	+	+	+
SA05	0.379	++	Strong Black	Producer	+	—	+	+	+	+	—	+
SA07	0.578	+++	Black	Producer	+	+	+	+	+	+	+	+
SA08	0.402	++	Black	Producer	+	+	+	+	+	+	+	—
SA13	0.491	++	Almost Black	Producer	—	+	+	+	+	+	+	+
SA14	0.586	+++	Almost Black	Producer	+	+	+	+	+	+	+	—
SA20	0.216	+	Bordeaux pink	Nonproducer	—	—	+	+	+	+	+	—
SA26	0.693	+++	Almost Black	Producer	+	+	+	+	+	+	+	—
SA28	0.339	++	Almost Black	Producer	+	+	+	+	+	+	+	—
SA32	0.219	+	Strong Black	Producer	+	+	+	+	+	+	+	+
SA38	0.785	+++	Almost Black	Producer	—	—	+	+	+	+	+	+
SA41	0.378	++	Almost Black	Producer	+	+	+	+	+	+	+	—
SA43	0.381	++	Strong Black	Producer	+	+	+	+	+	+	+	+
SA48	0.277	+	Strong Black	Producer	+	+	+	+	+	+	+	+
SA49	0.667	+++	Strong Black	Producer	+	+	+	+	+	+	+	—
SA54	0.329	++	Black	Producer	+	+	+	+	+	+	+	+
SA62	0.692	+++	Strong Black	Producer	+	+	+	+	+	+	+	+
SA70	0.398	++	Strong Black	Producer	+	+	+	+	+	+	+	+
SA77	0.275	+	Almost Black	Producer	+	—	+	+	+	—	+	+
SA78	0.376	+	Strong Black	Producer	+	+	+	+	+	—	+	+
SA86	0.827	+++	Strong Black	Producer	+	+	+	+	+	—	+	—
SA88	0.618	+++	Strong Black	Producer	—	—	—	—	—	—	+	—
SA93	0.790	+++	Black	Producer	—	—	+	+	+	—	—	—

*: indicating the varied adhering ability of *S. aureus* isolates, where strong black colonies; strong biofilm producer, black colonies; moderate biofilm producer, almost black colonies; weak biofilm producer, Bordeaux pink colonies; non – biofilm producer, also +: weak biofilm producer. ++: moderate biofilm producer. +++: strong biofilm producer. [1]: ODC: mean + 3 standard deviation of negative control in microplate. ODC=0.139, 2ODC=0.278, 4ODC=0.556

DISCUSSION

All 100 *S. aureus* isolates were branded as Gram positive; further cultural characteristics and biochemical test results were stated in Table 3. Out of these isolates, 91 isolates were coagulase positive *S. aureus*, and 9 were recorded as coagulase negative. The conventional biochemical tests and PCR method match 91% of results (9% non—reluctant due to analysis conditions and sample preparation) between the methods. This possibly will be due to concealing of the clumping factor by the capsular polysaccharides or due to misidentification of certain clumping factor producing CoNS; by this means, lessening its efficiency, reliability and sensitivity [(26)].

One more a lot test used for the *S. aureus* identification is the DNase test. In the existing study, all *S. aureus* isolates were positive for DNase activity. Parallel results have been reported previous too with no clarification for these findings and like this, requires confirmation using an additional test.

In our study stated that 78 *S. aureus* isolates have been able to hydrolyze the gelatin which mean have the gelatinase enzyme. However, 81, 51, and 48 isolates of *S. aureus* secret protease, lipase enzymes, and nonwhite pigmented colonies, respectively. Among the 100 isolates of *S. aureus* showed β–hemolytic, γ–hemolytic and α–hemolytic properties. Seventy one isolates of *S. aureus* have the ability of β–hemolysin and only 2 isolates exoressed α–hemolysin, and 27 isolates expressed the γ–hemolytic activity. Hemolytic activity among 57 different clinical sources of *S. aureus* exhibited strong hemolytic activities because of formation of large radius of clear zone on sheep blood agar and complete hemolysis; and five clinical strains which had no ability to blood hydrolysis on blood agar [27]. All isolates of *S. aureus* were undergone to PCR using universally conserved 16S rRNA and *nuc* genes, specifically designed primers were used for discrimination of *S. aureus* isolates (Table 2), and all isolates (100) were positive for both genes (Figure 1). Antimicrobial susceptibility carried out for 16 antimicrobials through disc diffusion methods, and the results are presented in Table 6. The highly resistant percentage was seen toward oxacillin (99%) followed by penicillin (97%) and the lowest resistant 1% was toward each of gentamycin, nitrofurantoin and trimethoprim–sulfamethoxazole. Based on the antimicrobial susceptibility reports by [28] among the 765 assessed *S. aureus* isolates, the maximum resistance rates were observed to penicillin (87.1%), followed by azithromycin (11.6%), erythromycin (11.2%) and clindamycin (9.7%). The mechanism basis of resistance to oxacillin and methicillin is through gain of a gene that encodes a homologue of the PBP2 called PBP2a which is not susceptible to drug deed. This is because of the serine active site of the TP of PBP2a which is not reachable to β–lactams [29].

Clinical *S. aureus* isolates were examined for biofilm formation *via* phenotypic and molecular assays and any possible association between biofilm formation and bacterial genetic lineage was investigated. *S. aureus* remains the most recurrently encountered bacterial pathogen and is responsible for a variety of slight to life threatening infections [30]. The facility of *S. aureus* to adhesion and from biofilm makes them more resistant against antibiotics. Bacterial virulence factor like adhesion factor plays an important role in infections associated with catheters. The ability of *S. aureus* to settle in artificial material is linked with two main mechanisms; production of polysaccharide slime, and adhesions for the host matrix proteins that are adsorbed onto the biomaterial surface. When the biofilm produced, it would be easy to run away from immune systems and to cause chronic infections. Although, for biofilm formation by *S. aureus*, PIA is important, this study found that more than one gene of *ica* operon i.e. *icaABCD*, *cna*, *atl*, *fnbA* and *fnbB* genes is related with biofilm production in this isolates.

Although, many genes and conditions are responsible to biofilm production also our results demonstrated that *icaA*, *icaB*, *icaC*, *icaD*, *cna*, *atl*, *fnbA* and *fnbB* genes have a critical role in biofilm creation. In this work we found that 89 out of 100 strains were biofilm producers developing almost black (*n=25*), black (*n=15*) or strong black colonies (*n=49*) on CRA plate, while in MTP assay, all isolates produce biofilm with generating weak (*n=8*), moderate (*n=71*) and strong biofilm production (*n=21*). Comparatively near results have been achieved elsewhere suggesting that 50% of clinical *S. aureus* isolates form biofilm [31]. Out of 100 bacterial isolates, formation of biofilm was recorded in 60% of isolates by CRA method, while PCR detection of biofilm related genes, *icaA* and *icaD*, revealed that both genes were present in 78% of isolates [7]. Discrepancy between phenotypic and genotypic methods for detection of biofilm producing among *S. aureus* has been reported earlier by [32]. Statistical analysis revealed a significant difference between the biofilm formation using the MTP assay (OD_{570}) and the CRA ($P< 0.001$). This discrepancy may be due to different mechanisms participate in *S. aureus* adherence on *in vitro* tests. Numerous studies have confirmed that *icaADBC* is necessary for biofilm formation by some *S. aureus* strains [33]. On the other hand,

adhesion to host cells needs genes such as *fnbA*, *fnbB,* and *cna* that encode microbial surface constituents knowing adhesive matrix molecules diverse from those involved in the adhesion on abiotic surfaces [34].

In the present study, the prevalence of the *icaC*, *icaD* and *cna* (96%) and *icaA*, *icaB*, *atl*, *fnbA* and genes were found to be 80, 72, 80 and 86% respectively, which may reflect their role in the pathogenicity of *S. aureus* isolates. However, *fnbB* was found in lower frequency (60%). The prevalence of twelve genes involved in production of biofilm explains that numerous factors may be effective in different steps of biofilm formation because all of the strains had ability to form biofilm in various levels but the incidence of genes was different. Further researches are considered necessary to elucidate the expression of these genes in *S. aureus* strains [35].

CONCLUSION

In the current study a significant association between presence of *icaA*, *icaB*, *icaC*, *icaD*, *can*, *atl*, *fnbA* and *fnbB* genes and biofilm formation were observed. Although all of the genes were not found simultaneously in all of the biofilm producer isolates, however, in the entire biofilm producer isolates (except one isolates) at least one of the related genes was detected. The ability of biofilm formation in isolates negative for one or more genes may be related to another biofilm associated genes.

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