Biofilm Formation of *Staphylococcus aureus* Isolated from Infected Wound

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Abstract
Background: *Staphylococcus aureus* has the ability to form biofilms, and causes significant mortality and morbidity in the patients with wounds. Our aim was to study the in vitro biofilm-forming ability of isolated *S. aureus*
patients and methods: one hundred clinical isolates of *S. aureus* were isolated from 350 pus samples using standard microbiological techniques. Biofilm formation ability of these isolates was detected phenotypically by tissue culture plate (TCP) method and congo red agar (CRA) and genotypically by detection of ica ABCD genes by PCR.
Results: The clinical isolates of *S. aureus* recovered from infected wounds exhibit a high degree of biofilm formation Biofilm formation was observed in (76 %), (74%) and (70%) isolates of *S. aureus* via TCP method CRA and genotypically, respectively.
Conclusion: This study illustrated that PCR method can be adopted as most suitable an reproducible method for detection of biofilm. CRA is qualitative, simple, inexpensive and easily reproducible method and convenient as screening method. TCP is semiquantitative method and remains a precious tool for in vitro screening of different biomaterial for the adhesive properties. Regular surveillance of biofilm formation by *S. aureus* leads to the early treatment of the wound infection.

Introduction
Staphylococcus aureus is an opportunistic pathogen implicated as the most common agent of skin and soft tissue infections. It can breach the skin barriers through the wound or surgical incision and cause infection. Furthermore, it has the ability to adhere to and form a biofilm on tissues or medical indwelling devices [1]. Biofilms are the aggregation of bacteria embedded in a self-produced extracellular matrix of exopolysaccharides (EPSs), proteins and some micromolecules such as DNA. They can form on both biotic and abiotic surfaces [2]. *S. aureus* initially adheres to a solid substrate, after which cell–cell adhesion occurs; the bacteria then multiply to form a multilayered biofilm encased in EPS. In fact, biofilm formation involves the production of polysaccharide intercellular adhesin, which depends on the expression of the intercellular adhesion (*Ica*ADBC) operon that encodes three membrane proteins (*Ica*A, *Ica*D and *Ica*C) and one extracellular protein (*Ica*B) [2]. Biofilm formation by *S. aureus* can lead to a delay in reepithelialization of the infected tissues, ultimately increasing healing time. *S. aureus* biofilms have been associated with chronic wounds like diabetic foot ulcer, pressure sores and venous ulcers. Detachment of matured biofilm of *S. aureus* is a prerequisite for the dissemination of wound infection [3].

• *Assay of biofilm production by S. aureus using Congo Red Agar (CRA)*: The isolates were cultured on CRA plates, prepared by adding 0.8 g of Congo red stain (Oxoid, UK) and 36 g of sucrose to 1 L of BHI (both from Oxoid, UK). After 24 h incubation at 37°C, isolates with red colonies were considered to be non-slime producing, and those with black colonies were considered to be slime- producing or biofilm-producers [5]**.**

• *Assay of biofilm production by S. aureus using* microtiter plate assay (MtP) [6]. Isolates from fresh agar plates were inoculated in trypticase soy broth with 1% glucose and incubated for 24 hours at 37°C in stationary condition and diluted (1 in 100) with fresh medium. Individual wells of sterile, polystyrene, flat-bottom tissue culture plates were filled with 0.2 ml aliquots of the diluted cultures, and only broth served as control to check sterility and non-specific binding of media. The tissue culture plates were incubated for 24 hours at 37°C. After incubation, the content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free floating planktonic bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate for 20 minutes and stained by crystal violet (0.1%). The plates were incubated at room temperature for 15 minutes, rinsed thoroughly and repeatedly with water. Crystal violet-stained biofilm was solubilized in 200 µL of 95 % ethanol (to extract the violet color), of which 125 µL were transferred to a new polystyrene microtiter dish, which was then read. Optical density (OD) of stained adherent bacteria was determined with ELISA autoreader ***(****Stat Fax 2100 autoreader)* at wave length of 545nm. Experiments for

Biofilms can resist antibiotic concentration 10-10,000 folds higher than those required to inhibit the growth of free floating bacteria [4].So, regular surveillance of biofilm formation by *S. aureus* and their antimicrobial resistance profile may lead to the early treatment of the wound infection. Therefore, our aim was to study the in vitro biofilm-forming ability of *S. aureus* isolated from wounds of hospitalized patients .

Patients and Methods

This study was conducted in Department of Medical Microbiology and Immunology, Faculty of Medicine, Sohag University. Pus from infected wounds were collected by sterile disposable cotton swabs. Samples were collected from patients admitted at Sohag University Hospitals from different surgical departments. All *Staphylococcus aureus* isolates were identified by, Gram staining (Gram positive cocci in grape like clusters) colony morphology (golden on nutrient agar ,beta hemolytic on blood agar and caused yellow discoloration on mannitol salt agar) and conventional biochemical tests (positive catalase and coagulase tests).

The following data were collected from patients:

1) Patients demographic data.

2) Possible risk factors as implants ,DM , use of broad-spectrum antimicrobials ,previous hospital admission and chronic diseases (other than DM) that affect wound healing like anemia, ischemia, renal and liver diseases

Phenotypic Detection of Biofilm Formation : Two phenotypic methods were used for detecting the biofilm production of the staphylococcal isolates; one qualitative (Congo red agar method) and another quantitative (Microtiter plate method).

ii. DNA amplification :The amplification reactions were prepared in a 25 µl volume containing the following; 12.5 µl PCR master mix *(Gene Direx)*, 7 µl Sterile Water,1.25 µl forward primer ,1.25 µl reverse primer and 3µl DNA. Each of the oligonucleotide primers specific for icaA ,ica B , ica C and icaD, respectively (see table 1 for the sequences) .The thermal amplification program for ica A and ica D included the following steps: an initial denaturation at 95ºC for 5 min; 50 cycles of amplification with 94ºC for 30 s (denaturation),55.5ºC for 30 s (annealing), 72ºC for 1 min extension); and then final extension at 72ºC for2 min. The thermal amplification program for ica B and ica C included the following steps: an initial denaturation at 95ºC for 5 min; 30 cycles of amplification with 94ºC for 1 min (denaturation),59ºC (ica B) and45 ºC(ica C) for 1min(annealing), 72ºC for 2.5 min extension); and then final extension at 72ºC for10 min.

iii. Detection of the amplified genes: 10 µl of the amplification products were electrophoresed on agarose gel along with molecular weight marker100 bp DNA ladder, and the presence or absence of any resulting bands was evaluated under ultraviolet transillumination.

each strain were performed in triplicate and repeated three times. To compensate for background absorbance, OD readings from sterile medium were averaged and subtracted from all test results., and average OD values of negative controls and samples were calculated separately. Optical density cut-off value (ODc) = average OD of negative control +3 standard deviation (SD) of negative control [6].

Interpretation of results was described as follows:[7]

1. OD ≤ ODc= Non biofilm producer (N).
2. ODc <OD ≤ 2ODc = Weak biofilm producer (WP).
3. 2ODc < OD ≤ 4ODc =Moderate biofilm producer (MP).
4. 4ODc < OD= Strong biofilm producer(SP).

Genotypic *detection of BiofilmFormation*

Simple qualitative polymerase chain reaction for detection of ica ABCD genes was done as follows:

i. DNA extraction (the boiling method):Few isolated colonies of overnight growth bacteria were suspended thoroughly in 50 µl sterile distilled water. The suspension was boiled in a water bath, for 10 min. It was centrifuged at 10000 rpm for 5 min, The supernatant was taken as a template and stored at -20° C [8].

Table (1): primers used in the study

Gene	Primer	Nucleotide Sequence	Amplicon size	Refer ence
Ica A	**Forward** **Reverse**	**5'-TCTCTTGCAGGAGCAATCAA -3'** **5'-TCAGGCACTAACATCCAGCA -3**	**188 bp**	**[9]**
Ica B	**Forward** **Reverse**	**5'- ATG GCT TAA AGC ACA CGA CGC -3'** **5'- TAT CGG CAT CTG GTG TGA CAG -3**	**526 bp**	**[10]**
Ica C	**Forward** **Reverse**	**5' TGCATTTTATCGATCAGGGC 3'** **5' CACTTCCTTTTCCAGGACG 3'**	**989 bp**	**[10]**
Ica D	**Forward** **Reverse**	**5'- ATA AAC TTG AAT TAG TGT ATT -3'** **5'- ATA TAT AAA ACT CTC TTA ACA -3**	**198 bp**	**[9]**

Results

The study included 350 patients with wound infections isolated from patients recruited from different departments. *Staphylococcus aureus* was isolated in 100 patients .

i. *Detection of Biofilm formation by phenotypic methods*:

- Biofilm formation by tissue culture plate method; 24% of *S.aureus* isolates were non biofilm producers and 76% were positive biofilm producers (9%weak,48%modeate and 19%strong)
- Biofilm formation by congo red method; 26% of *S.aureus* isolates were non biofilm producers and 74% were positive biofilm producers (29%modeate and 45%strong). Congo red has statistically significant correlation with TCP ***(p value =0.001***) (Table 2).

Table(2): Distribution of the studied patients according to the results of Congo red and TCP test

Congo red test	TCP test				P-value
	Non NO. (%)	Weak NO. (%)	Moderate NO. (%)	Strong NO. (%)	
Non /Weak	12(46.2%)	3(11.5%)	9(34.6%)	2(7.7%)	0.001*
Moderate	3(10.3%)	4(13.8%)	20(69.0%)	2(6.9%)	
Strong	9(20.0%)	2(4.4%)	19(42.2%)	15(33.3%)	

ii. Detection of Biofilm formation by genotypic method (PCR ; detection of ica genes)

- Regarding presence of one or more of ica genes in S.aureus strains; 70% positive and 30% negative
- We found that there Ica A was present in 23% of isolates, Ica B was present in 11% of isolates , Ica C was present in 9% of isolates and Ica D was present in 70% of isolates.

iii. comparison between TCP, congo and genotypic method for detection of biofilm formation

- On comparison between TCP and genotypic method for detection of biofilm formation; sensitivity of TCP in comparison to PCR was 97.1%, specificity was 73.3 %, positive predictive value was 89.5% and negative predictive value was 91.7%. Two isolate was positive biofilm producer by PCR and negative biofilm producer by TCP. Eight isolates were non biofilm producers by PCR and positive biofilm producers by TCP method. There was high statistically significant relation between TCP and PCR mehods for detection of biofilm ***(p value < 0.0001)*** (table 2,3).
- On comparison between congo red and genotypic method for detection of biofilm formation ;sensitivity of congo red method in comparison with PCR was 77.1%, specificity was 33.3%, positive predictive value was 73% and negative predictive value was 38.5%. Sixteen isolates was positive biofilm producer by PCR and negative biofilm producer by congo red method. Twenty isolates were non biofilm producers by PCR and positive biofilm producers by congo red method . There was statistically significant relation between CRA and PCR methods for detection of biofilm ***(p value =0.008)*** (table 3,4)

Table (3): Comparison between the results of Congo red and TCP test and PCR.

	Biofilm formation genotypiclly				P-value
	No (-ve) N.= 30(30.0%)		Yes (+ve) N.=70(70.0%)		
TCP					
No (-ve)	22	(73.3)	2	(2.9)	<0.0001*
Yes (+ve)	8	(26.7)	68	(97.1)	
Congo red					
No (-ve)	10	(33.3)	16	(22.9)	0.008*
Yes (+ve)	20	(66.7)	54	(77.1)	

Table (4): Sensitivity, specificity, positive predictive value (PPV), negative predicative value (NPV) of TCP and Congo red

	Sensitivity	Specificity	PPV	NPV
TCP	97.1	73.3	89.5	91.7
Congo red	77.1	33.3	73	38.5

iv. Some the possible risk factors for biofilm formation by *S.aureus* in infected wounds were studied and results shown in table 5.

Table (5): comparison between biofilm forming and non biofilm forming groups regarding possible risk factors.

	Biofilm formation		P-value
	Yes N.=70(70.0%)	No N.= 30(30.0%)	
Age Mean± S.D. Median(Range)	35.1±21.6 31.0(4.0 -70.0)	37.0±19.0 40.0(4.0-72.0)	0.585
Sex Male (%) Female (%)	41(83.7%) 29(56.9%)	8(16.3%) 22(43.1%)	0.003*
Bed Sores No (%) yes (%)	63(67.7%) 7 (100%)	30 (32.3%) 0 (0.0%)	0.001*
Burn No (%) yes (%)	68(75.6%) 2 (20.0%)	22(24.4%) 8(80.0%)	0.099
Diabetic foot No (%) yes (%)	55 (67.1%) 15(83.3%)	27(32.9%) 3(16.7%)	0.173
Surgical Wound No (%) yes (%)	26(65.0%) 44(73.3%)	14(35.0%) 16(26.7%)	0.373
Traumatic Wound No (%) yes (%)	68(71.6%) 2(40.0%)	27(28.4%) 3(60.0%)	0.158
Diabetes Mellitus No (%) yes (%)	51(68.0%) 19(76.0%)	24(32.0%) 6(24.0%)	0.450
Foreign body No (%) yes (%)	43(64.2%) 27(81.8%)	24(35.8%) 6(18.2%)	0.070
Previous hospital admission No (%) yes (%)	24(53.3%) 46(83.6%)	21(46.7%) 9(16.4%)	0.001*
Use of broad spectrum antibiotics No (%) yes (%)	22(51.2%) 48(84.2%)	21(48.8%) 9(15.8%)	0.001*
Steroid No (%) yes (%	64(91.4%) 6(8.6%)	25(83.3%) 5 (16.7%)	0.298
Chronic disease No (%) yes (%)	23(32.9%) 47(67.1%)	30(100%) 0(0.0%)	<0.0001 *

P- value was calculated by Chi square test and Fisher's Exact Test * Statistically significant

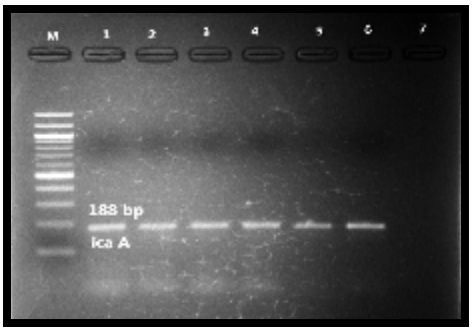

Figure (1): Electrophoresis of PCR products with primers for ica A. Lane M, 100 bp molecular weight marker; from lane1,to lane 6, 188-bp bands from ica A positive samples; lane 7, negative control.

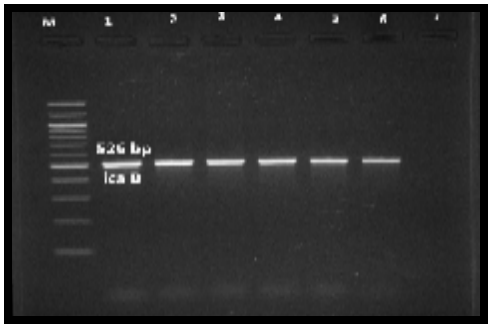

Figure (2): Electrophoresis of PCR products with primers for ica B. Lane M, 100 bp molecular weight marker; from lane1,to lane 6, 526-bp bands from ica B positive samples; lane 7, negative control

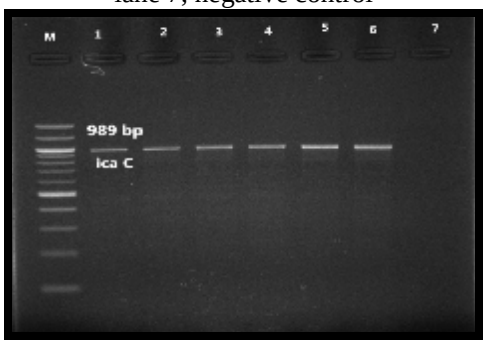

Figure (3): Electrophoresis of PCR products with primers for ica C. Lane M, 100 bp molecular weight marker; from lane1,to lane 6, 989-bp bands from ica C positive samples; lane 7, negative control.

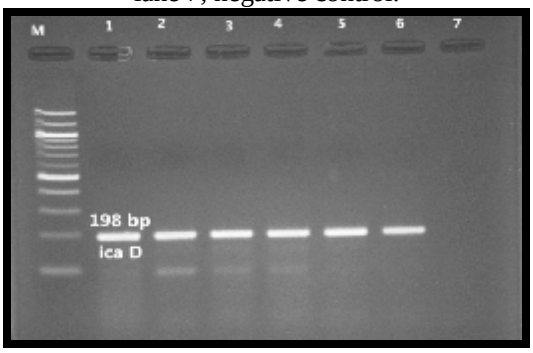

Figure (4): Electrophoresis of PCR products with primers for ica D. Lane M, 100 bp molecular weight marker; from lane1,to lane 6, 198-bp bands from ica D positive samples; lane 7, negative control

Discussion

Biofilm formation by tissue culture plate method; 24% of *S.aureus* isolates were non biofilm producers and 76% were positive biofilm producers (19% strong 48% modeate, and 9%weak). Other studies that investigated biofilm formation by *S.aureus* in wounds showed also high prevalence of biofilm formation ***Neopane et al.,(2018)*** [2] (69.8%;6.97 strong 27.90% moderate and 34.88% weak) *and* ***Yazdani et al*.(2006)** [11] (52%). In another study in Egypt, ***Gad et al.* (2009)**[12] reported higher prevalence of biofilm formation by *S.aureus* but in urine samples in-patients undergoing ureteral catheterization (83.3%; 66.7% strong biofilm 16.7% moderate and 16.7% non or weak). A lower rate of biofilm formation was demonstrated by ***Nasr et al. (2012)*** [13] where 46% of *S.aureus* isolates produce biofilm by TCP assay ; 26% strong producers, 12% moderate and 8% weak biofilm producers. Variation may be due to different type of samples, presence of foreign body, different growth conditions and the use of various sugar supplementations for biofilm formation in *staphylococci*.

Our data, using samples isolated from wound and pus, are in coordenence to that of a previous report that showed 66.67% biofilm formation in the blood samples ***(Poudel et al., 2015)*** [14] The potential for biofilm formation in wounds and pus may be similar to that in the blood. Biofilm formation depends on many factors such as environment, availability of nutrients, geographical origin, types of specimen, surface adhesion characteristics and genetic makeup of the organism[15]. These factors may have affected the data and contributed to the high prevalence observed in the present study. However, it is not known as to how these factors are involved. Biofilms can form on any wound when planktonic bacteria are not eliminated by the host's immune system or by exogenous antimicrobial agents. In addition, mutations in *Ica* and regulatory genes have been associated with reduced capacity of *S. aureus* to form biofilms[16].Taken together, these factors may have affected the results in the present study.

Biofilm formation by congo red method; 26% of *S.aureus* isolates were non biofilm producers and 74% were positive biofilm producers (29% modeate and 45%strong). ***Namvar (2013)*** [17]. and ***Nasr et al., (2012)*** [13]. also reported 65% positive results with congo red agar. However ***Taj et al.(2012)*** [18]. reported that only four isolates (3.4%) were positive by CRA test .

Congo red had statistically significant correlation with TCP ***(p value =0.001)***.Our findings are contradictory with ***Nasr et al.(2012)*** [13] who reported that CRA method showed little correlation with MTP assay where only (20%) of the isolates were positive by both the MTP and CRA methods. A low correspondence between both methods was also demonstrated by

Marthur et al.(2006) [19].On the other hand, better correlation between both methods were reported by other investigators where all *staphylococci* positive by one test were also positive by the other ***(Cafiso et al. 2004)*** [20].Environmental factors like sugars (glucose or lactose) or proteases present in the growth medium, surface area, type of surface (rough/smooth), porosity, charge of the surface and the genetic makeup of the *S. aureus* isolate affect biofilm formation ***(Lotfi et al.,2014)*** [21].

In our study we detect biofilm formation genotypically by simple qualitative PCR for detection of ica genes (ica A, ica B, icaC and icaD) as indicator for biofilm formation. PCR is the most widely used technique in molecular biology because it is simple, sensitive, specific and very efficient compared to other methods[22]**.**In the present study, 70 strains (70%) were found to contain one or more of these genes and 30 strains (30%) were negative for all genes. We found that there Ica A was present in 23% of isolates, Ica B was present in 11% of isolates , Ica C was present in 9% of isolates and Ica D was present in 70% of isolates. ***(Diemond-Hernández et al.,2010)*** [10] detect ica A in 10.3% and ica D in 97.5% of *S.aureus* isolates and didn't detect ica B or ica C. ***Al-Mtory et al. (2016)*** [22] and ***Mirzaee et al., (2014)*** [23] reported higher percentage than our study. ***Al-Mtory et al. (2016)*** [22] demonstrate that the prevalence of icaA, icaB , icaC and icaD were 95.8%, 91.6% ,45.8% and 95.8% respectively. In a study of ***Mirzaee et al. (2014)*** [23] ,the prevalence of icaA, icaB , icaC and icaD were 51.6%, 45.1% ,77.4% and 80.6% respectively. ***Torlak et al., (2017)*** [24] and ***Tekeli et al.(2016*)** [25] reported high prevalence of *ica* genes among *S. aureus where all* isolates of *S. aureus* were reported to possess ica A and ica D genes . ***Arciola et al. (2001)***[9] and ***Gad et al. (2009)***[12]who detected ica A and ica D genes in all biofilm *S. aureus* isolates. .The inconsistency across various studies might be due to heterogeneity in the origins of bacteria such as genetic characterization, source of isolation and environmental conditions.

On comparison between TCP and genotypic method for detection of biofilm formation; sensitivity of TCP in comparison to PCR was 97.1%, specificity was 73.3 %, positive predictive value was 89.5% and negative predictive value was 91.7%. Most studies on biofilm agreed with our study and reported high sensitivity ,specificity , positive predictive value and negative predictive value of TCP ***Mirzaee et al. (2014)*** [23] . ***Aricola et al. (2002)***[9] ***, Gad et al. (2009)*** [12] *and* ***Oliveira and Cunha Maria de Lourdes (2010)*** [26] **.**

In our study, two isolates was positive biofilm producer by PCR and negative biofilm producer by TCP this could depend on the culture condition in MTP causing variability depending on the type of incubation medium, so some strains appear negative because their phenotype is not completely expressed in TSB broth. Eight isolates were non biofilm producers by PCR and positive biofilm producers by TCP method. There was high

statistically significant relation between TCP and PCR methods for detection of biofilm ***(p value < 0.0001).*** This is in coordenence with ***Mirzaee et al. (2014)*** [23]. also found that one of the *S.aureus* isolates included in their study was negative for all of *ica* genes but still produced biofilm as shown by MTP method, suggesting that the difference between the phenotypic and the genotypic characterization of the strain may be explained by an alternative PIA-independent mechanism for biofilm formation in this isolate. 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 (IS256), in *icaA* gene and causing its inactivation [27] . On comparison between congo red and genotypic method for detection of biofilm formation; sensitivity of congo red method was 77.1%, specificity was 33.3%, positive predictive value was 73% and negative predictive value was 38.5%. Sixteen isolates was positive biofilm producer by PCR and negative biofilm producer by congo red method. Twenty isolates were non biofilm producers by PCR and positive biofilm producers by congo red method .54 isolates were positive biofilm producers of 70 isolates positive by PCR. There was statistically significant relation between CRA and PCR methods for detection of biofilm ***(p value =0.008)***. ***Solati et al.(2015)*** [28]***, Aricola et al. (2005)*** [29] *and* ***Terki et al. (2013)*** [30] demonstrated also agreement between results of between CRA and PCR. In our study, positivity at the CRA plate test did not always correlate with the presence of ica A and ica D genes, in accordance with ***El-Amin et al.(2015)*** [31] who demonstrated that 2% of strains with ica genes did not express phenotype. ***Liberto et al.(2007)*** [32] hypothesize a translational or post-translational regulation with production of proteins with low or absent activity, associated with an absent phenotype. As Slime production and association in biofilm are two parameters of great complexity: they are highly correlated with the environment. Indeed, anaerobiosis and low concentrations of iron strongly increase biofilm expression ***(Baldassarri et al.,(2001)*** [33] and ***Cramton et al., (2001)*** [34] . On the other hand ,recent studies highlighted the role of phenol- soluble modulines that can control the passage from biofilm phase to non-biofilm phase, with subsequent dissemination **(Yao et al., 2005)** [35]**.** More- over, glucose concentration and, even more, glucose uptake of a particular strain ,and/or a peculiar phase of the growth curve ,can influence ica operon transcription and biofilm expression ***(Dobinsky et al., 2003)*** [36].

In contrast to this study ***Nasr et al.(2012)*** [13] reported low sensitivity (31.25%) and specificity (47.05%) of CRA method in comparison to genotypic method and don't recommended it for detection of biofilm formation by staphylococcal clinical isolates. ***Oliveira and Cunha Maria de Lourdes, (2010)*** [26] study showed higher sensitivity (89%) and specificity(100%) of CRA method in comparison to ica genes . However, these authors concluded that CRA might be imprecise in the identification of positive isolates when compared to molecular analysis of the genes involved in biofilm production.

Regarding to studying some the possible risk factors for biofilm formation by *S.aureus* in infected wounds our study revealed that; The mean age ±SD was 35.1±21.6 for cases and 37.0±19.0 for controls with

(*P value = 0.585*). The median age was 31 years for cases and 40 for controls . The range for age was (4-70) for cases and (4 - 72) for controls which is statistically insignificant so no relation between age of the patients and biofilm formation by *S.aureus* in infected wounds. These results are in agreement with ***Shakibaie et al.(2015)*** [37] and ***Cha et al., (2013)*** [38] who found no relation between age of the patients and biofilm formation(***p value= 0.343*** and ***0.203*** respectively).

The sex distribution among cases was 41 males representing (83.7%) of all males included in the study and 29 females representing (56.9%) of all females included in the study, while the controls was 8 males representing (16.3%) of all males included in the study and 22 females representing (43.1%) of all females included in the study with ***(P value 0.003)*** which is statistically significant so there was significant relation between male gender and biofilm formation by *S.aureus* in infected wounds .This is in agreement with ***Cha et al. (2013)*** [38] *and* ***Taj et al. (2011)*** [18] and showed that gender had no relation with biofilm formation ***(p value 0.990*** and ***0.476 respectively).***

Regarding to the type of wound of studied population; 60(60%) *S.aureus* isolates were from infected surgical wounds, 18(18%) isolates from infected diabetic foot, 10 (10%) from infected bed sores ,7(7%) from infected burn wounds and 5(5%) from infected chronic wounds. There was a strong relationship between biofilm formation by *S.aureus* and bed sore infections ***(P value is <0.05). Abarna et al., (2017)*** [39] found no relation between type of wound and biofilm formation.

DM impacts the immune system and impair wound healing and impaired perfusion and tissue oxygenation as a result of the microvascular changes associated with DM this leads to higher possibility of infection and biofilm formation[40]. Yet, In our study there was no relation between biofilm formation and DM , the same was found by ***Luther et al.(2018*)** [41]. This may be the due to low number of diabetic patients enrolled in our study (~25%) ; thus, limiting the power of the analysis.

In general, implantation of medical devices (e.g., materials for wound stabilization, catheters, and joint prosthetics) has been frequently associated with the production of biofilms and subsequent infections***(Arciola et al., 2015)*** [42] and ***(Zalipour et al., 2016)*** [43]**.** Therefore, it was surprising that the presence of medical hardware was not statistically significant in our study . One explanation could be the low number of wounds that had implantation of medical hardware (~23%) ; thus, limiting the power of the analysis. Results of ***Luther et al.(2018)*** [41] and ***Akers et al.(2014)*** [44] are similar to our study.

There was highly significant relation between previous hospital admission and biofilm formation ***Luther et al.(2018*)** [41] ***Shakibaie et al. (2014)*** [37] and ***Cha et al. (2013)*** [38] reported the same results while ***Abarna et al.(2017)*** [39] found no difference between biofilm forming and nom forming groups.

Using of broad spectrum antibiotics and presence of chronic diseases (other than DM) that affect wound healing -like anaemia ,ischemia and malnutrition - have highly significant relation with biofilm formation by *S.aureus* in infected wounds **(*p value <0.001*) . *Luther et al.(2018)*** [41] and ***Abarna et al., (2017)*** [39] reported no difference between biofilm forming and non biofilm regarding to these comorbidities while groups **Taj et al. (2011)** [18] results

were the similar to this study. The discrepancy in clinical risk factors affecting biofilm formation may be due to different size and of the samples and difference between in vitro and in vivo biofilm formation and accuracy in recording data of the patients.

Conclusion

This study illustrated that biofilm formation is an important cause of antibiotic resistance in *S.aureus* isolated from infected wounds. Our results have confirmed data presented by other authors in that the presence of icaADBC operon genes is associated with biofilm formation .Therefore, both genotypic and phenotypic methods improve identification biofilm ability by *S.aureus*. PCR method can be adopted as most suitable an reproducible method for detection of biofilm. CRA is qualitative, Simple, inexpensive and easily reproducible method and convenient as screening method. TCP is semiquantitative method and remain a precious tool for in vitro screening of different biomaterial for the adhesive properties .Each method has its advantages and drawbacks, as well as their specific indication. On the other hand, the biofilm-forming ability of some strains in the absence of icaABCD genes highlights the importance of further genetic investigations of ica independent biofilm formation mechanisms.

Regular surveillance of biofilm formation by *S. aureus* and their antimicrobial resistance profile leads to the early treatment of the wound infection.

Reference

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