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ORIGINAL ARTICLE

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.* ***Objective:** Our aim was to study the in vitro biofilm-forming ability of isolated S. aureus.* ***Methodology**: 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 exhibited a high degree of biofilm formation. Biofilm formation was observed in (76%), (74%) and (70%) of S. aureus isolates via TCP method CRA and genotypically, respectively.* ***Conclusion**: This study illustrated that PCR method can be adopted as the most suitable and reproducible method for detection of biofilm. CRA is qualitative, simple, inexpensive and easily reproducible and convenient as a 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 the most common agent of skin and soft tissue infections. Also, it is able to form a biofilm on tissues or medical indwelling devices [1].

Biofilms are the aggregation of bacteria enclosed in extracellular matrix of exopolysaccharides (EPSs). They can form on both biotic and a biotic surfaces [1]. *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. 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 in infected wounds can lead to a delay in its reepithelialization, ultimately increasing healing time. Biofilm protects microoganisms from external environment, host immunity and antibiotic therapy [2].So, regular surveillance of biofilm formation by *S. aureus* and their antimicrobial resistance profile may lead to early treatment of wound infection. Therefore, our aim was to study the in vitro biofilm-forming ability of *S. aureus* isolated from wounds.

METHODOLOGY

This study was conducted in the Department of Medical Microbiology and Immunology, Faculty of Medicine, Sohag University. The study was carried out after having approval from the Ethics Committee. A written consent from all the patients included in the study was taken before initiation of the study. Pus samples 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).

Phenotypic Detection of Biofilm Formation:

- *Assay of biofilm production by S. aureus using Congo Red Agar (CRA)* [3].
- *Assay of biofilm production by S. aureus using* microtiter plate assay (MtP) [4]:

Optical density (OD) of stained adherent bacteria was determined with ELISA autoreader ***(****Stat Fax 2100 autoreader)* at wave length of 545nm. Experiments for each strain were performed in triplicate. 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 [4].
Interpretation of results was described as follows:[5]

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 icaABCD genes was done as follows:

DNA extraction (the boiling method):

Few isolated colonies of overnight growth bacteria were suspended thoroughly in 50µl 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[6].

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 ,icaB , icaC and icaD, respectively (see table 1 for the sequences) .The thermal amplification program for icaA and icaD as mentioned by Arciola et al. [7] *and for* icaB and icaC as mentioned by Diemond et al.[8].

Detection of the amplified genes:

10µl of the amplification products were electrophoresed on agarose gel along with molecular weight marker100 bp DNA ladder*(Bio Labs)*., and the presence or absence of any resulting bands was evaluated under ultraviolet transillumination.

Table 1: primers used in the study

Gene	Primer	Nucleotide Sequence	Amplicon size	Reference
IcaA	Forward Reverse	5'-TCTCTTGCAGGAGCAATCAA -3' 5'-TCAGGCACTAACATCCAGCA -3	188 bp	[7]
IcaB	Forward Reverse	5'- ATG GCT TAA AGC ACA CGA CGC -3' 5'- TAT CGG CAT CTG GTG TGA CAG -3	526 bp	[8]
IcaC	Forward Reverse	5' TGCATTTTATCGATCAGGGC 3' 5' CACTTCCTTTTCCAGGACG 3'	989 bp	[8]
IcaD	Forward Reverse	5'- ATA AAC TTG AAT TAG TGT ATT -3' 5'- ATA TAT AAA ACT CTC TTA ACA -3	198	[7]

RESULTS

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

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%moderate 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% moderate and 45% strong). Congo red has statistically significant correlation with TCP ***(p value =0.001***) (Table 2).

Table 2: Comparison between 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%)	

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.
- IcaA was present in 23% of isolates, IcaB was present in 11% of isolates, IcaC was present in 9% of isolates and Ica D was present in 70% of isolates (Figures 1-4).

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 isolates were positive biofilm

producers 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 a high statistically significant relation between TCP and PCR methods for detection of biofilm ***(p value < 0.0001)*** (Table 3).

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)	

– 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 4)

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

Some of 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

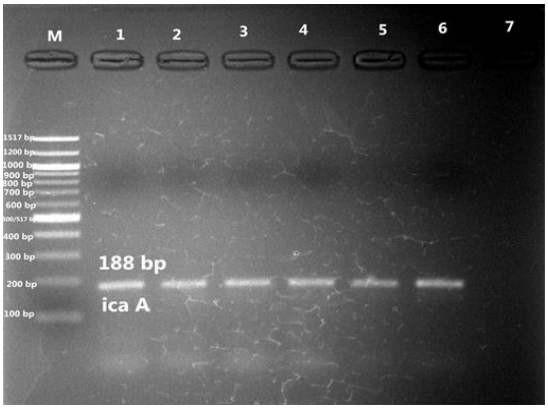

Fig. 1: Electrophoresis of PCR products with primers for icaA. Lane M, 100 bp molecular weight marker; from lane1,to lane 6, 188-bp bands from icaA positive samples; lane 7, negative control.

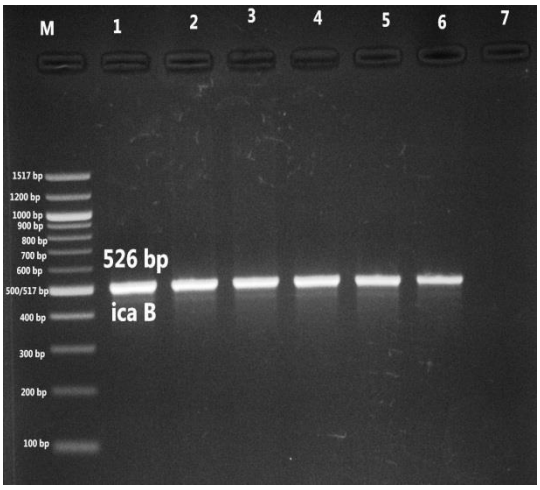

Fig. 2: Electrophoresis of PCR products with primers for icaB. Lane M, 100 bp molecular weight marker; from lane1,to lane 6, 526-bp bands from icaB positive samples; lane 7, negative control

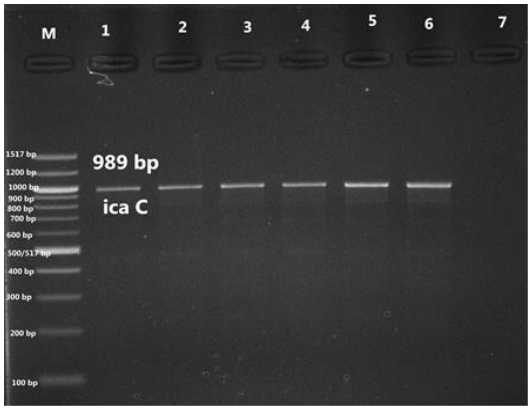

Fig. 3: Electrophoresis of PCR products with primers for icaC. Lane M, 100 bp molecular weight marker; from lane1,to lane 6, 989-bp bands from icaC positive samples; lane 7, negative control.

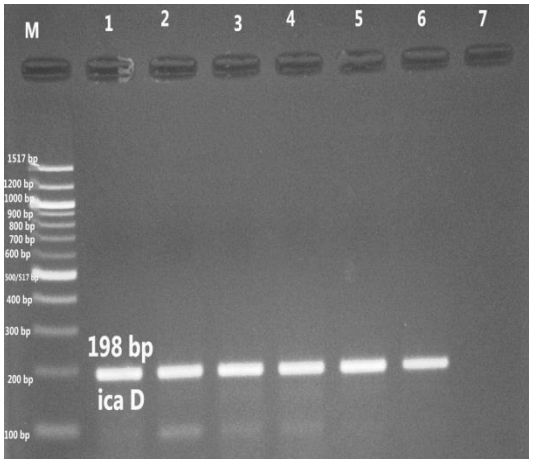

Fig. 4: Electrophoresis of PCR products with primers for icaD. Lane M, 100 bp molecular weight marker; from lane1,to lane 6, 198-bp bands from icaD positive samples; lane 7, negative control

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% moderate, and 9%weak). Another study that investigated biofilm formation by *S.aureus* in wounds showed also high prevalence of biofilm formation (69.8%;6.97 strong 27.90% moderate and 34.88% weak) [2]. A lower rate of biofilm formation was demonstrated by Nasr et al. [9] where 46% of *S.aureus* isolates produce biofilm by TCP assay; 26% strong producers, 12% moderate and 8% weak biofilm producers.

Biofilm formation by congo red method 26% of *S.aureus* isolates were non biofilm producers and 74% were positive biofilm producers (29% moderate and 45% strong). Nasr et al.[9] also reported 65% positive results with congo red agar. However Taj et al. [10] reported that only four isolates (3.4%) were positive by CRA test . 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*.

Congo red had statistically significant correlation with TCP *(p value =0.001)*.Our findings are contradictory with Nasr et al.[9] 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. Recently better correlation between both methods was reported by other investigators[11]. 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 make up of the *S. aureus* isolate affect biofilm formation [12].

In our study we detect biofilm formation genotypically by simple qualitative PCR for detection of ica genes (icaA, icaB, 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[8].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 IcaA was present in 23% of isolates, IcaB was present in 11% of isolates , Ica C was present in 9% of isolates and IcaD was present in 70% of isolates. Diemond-Hernández et al.[8] detect icaA in 10.3% and ica D in 97.5% of *S.aureus* isolates and didn't detect icaB or icaC. In a study of Mirzaee et al. [13] ,the prevalence of icaA, icaB , icaC and icaD were 51.6%, 45.1% ,77.4% and 80.6% respectively which is more than our study. Arciola et al. [7] and Gad et al. [14] detected icaA and icaD 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[13,14,20].

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.[13] 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 [14].

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. Fifty five 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)*. Terki et al.[15] 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.[16] who demonstrated that 2% of strains with ica genes did not express phenotype. 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[17].

In contrast to this study Nasr et al.[9] 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, [18] 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; Although 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 [2], in our study there was no relation between biofilm formation and DM , the same was found by Luther et al.[1]. 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 [12]. 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. [1] are similar to our study.

There was highly significant relation between previous hospital admission and biofilm formation Luther et al. [1] reported the same results while Abarna et

al.[19] 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.[1] and Abarna et al.[19] reported no difference between biofilm forming and non biofilm regarding to these comorbidities while groups .Taj et al. [10] 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.

CONCLUSIONS AND RECOMMENDATIONS

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.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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