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Biofilm-Producing Staphylococci, Genotypic and Phenotypic Study

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Staphylococci are incriminated in many of biofilm-related infections and considered main causes of hospital-acquired infections. The present study was conducted to evaluate biofilm-forming capacity and the presence of both *icaA* and *icaD* genes among Staphylococcal strains isolated from patients attending different Egyptian Hospitals. 250 Staphylococcal strains were isolated from 520 different clinical samples collected. Out of the 250 Staphylococcal strains, 160 were coagulase-positive staphylococci (CPS) and 90 were coagulase-negative staphylococci (CNS). All strains were studied for biofilm production using Standard Tube (ST) method, Congo Red Agar (CRA) method, and Tissue Culture Plate (TCP) method. Staphylococcal strains isolated from sputum and urine samples were more able to produce biofilms than strains isolated from other samples. Both CPS and CNS produced biofilm by the three mentioned methods in different percentages. CRA method revealed that 82.5% (132/160) of CPS strains were biofilm producers while by using ST method, the results were 64.3% (103/160). For CNS, the percentages were 71.1% (64/90) and 55.5% (50/90) using CRA and ST respectively. Screening by TCP revealed that 85% (136/160) of CPS strains and 84% (76/90) of CNS were biofilm producers. Effect of some physiological solutions on biofilm production was studied. Addition of 1% glucose increased biofilm formation while addition 4% NaCl decreased biofilm formation for both CPS and CNS. Different pH levels greatly affected biofilm production. pH 7.2 was the optimum for biofilm production. Presence of bap (biofilm-associated protein) was studied using poly acrylamide gel electrophoresis. The genes responsible for biofilm formation are *icaA* and *icaD* and could be detected by PCR technique. Detection of *icaA* and *icaD* genes agreed with TCP results. TCP method was found to be highly sensitive, accurate and has the advantage of being a quantitative model and can be used to study the adherence degree of staphylococci on biomedical devices. The results of TCP agree with the results of PCR. As a conclusion: The ability of Staphylococcal isolates to form biofilm in vitro appears to be an indication of a virulence trait that enhances the ability of isolates to cause infections. In addition, the results prove the role of *ica* genes and phenotypic variability of biofilm production as virulence factors in Staphylococcal infections.

Key words: Staphylococci, biofilm infections, genes.

Biofilm is an important microbial virulence factor in staphylococci. Microbial biofilms are communities of sessile microorganisms formed by cells that are attached to each other and embedded in a hydrated matrix of extracellular polymeric substances and are now regarded as the predominant mode of microbial life in nature and disease[1]. Bacteria prefer to live in biofilms to increase their survival. Adherent bacterial communities embedded in a self-produced matrix confer an unusually high resistance to the microorganisms living within them[2,3]. Biofilm formation is considered to be a two-step process in which the bacteria first adhere to a surface mediated by a capsular antigen followed by

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multiplication to form a multilayered biofilm, which is associated with production of polysaccharide intercellular adhesin (PIA)[4]. The intercellular adhesion (*ica*) locus consisting of the genes *icaADBC* encodes the proteins mediating the synthesis of PIA in Staphylococcal species[5,6]. Biofilms are very hard to eradicate and are responsible for a significant number of nosocomial and device-related infections[7] . The protein components of Staphylococcal biofilms are proteins intervene at different stages of the biofilm formation, with certain proteins contributing to the biofilm accumulation and others mediating the primary attachment to the surfaces[8]. Of all human infections up to 60% can be assigned to and any foreign medical implant, such as prosthetic joints, pacemakers and catheters, can become colonized with sessile bacteria, this results in increased biofilm-related morbidity and mortality[6,9]. Staphylococci are currently the most common cause of nosocomial infections. Opportunistic *S.aureus* is involved in native valve endocarditis, otitis media and all kinds of infections of implanted devices[10].

MATERIALS AND METHODS

Two hundred and fifty of Staphylococcal strains were isolated from 520 clinical samples collected from patients attending different Egyptian Hospitals. The isolated strains were identified using API strips and standard methods[11,12].

Biofilm production

The ability of the isolated Staphylococcal strains to form biofilms was studied using different methods[13-15] as follow:

Congo Red Agar (CRA) method

Using brain heart infusion broth 37 g/l, sucrose 50 g/l, agar 10 g/l, and Congo red 0.8 g/l. The Congo red stain was sterilized separately by autoclave and added to the agar at 55ºC. Agar plates were inoculated and incubated for overnight. Production of black colonies is considered as slime positive while those showed pink colonies are considered slime negative.

Standard Tube (TM) method

A loopful of organisms from a single colony in pure culture on tryptic soy agar plate was inoculated into 5 ml of trypticase soy broth. The inoculated tubes were incubated at 37ºC. After 24 hrs, the contents were decanted. The tubes were then stained with 1% safranin for 7 min. A positive result was indicated by the presence of an adherent film of stained material on the inner wall of the tube.

Tissue Culture Plate (TCP) method

A quantitative determination using tissue culture plates with 96 flat bottomed wells. Each well was filled with 0.2 ml of 10^5 CFU/ml of a bacterial suspension in TSB. After 48 hrs incubation at 37 ºC, the contents were aspirated and the plates were washed twice with phosphate-buffer saline (PBS, pH 7.2). The wells were then stained with 0.25% crystal violet for 30 sec. The plates were read by ELISA reader (BioTek, ELx808) at 490 nm. Sterile TSB was used as a negative control. The values of optical density were then averaged. A 3-grade scale was used to evaluate the strains slime producing ability; (-): ODs < 0.500; (+): ODs 0.500-1.500; (++): ODs >1.500.

Effect of some physiological solutions on biofilm production

The effect of some physiological solutions such as glucose 1%, NaCl 4% and different pH levels on biofilm production was studied[15].

Detection of *icaA* and *icaD*

Detection of *icaA* and *icaD* was performed according to the previous method[16]. Bacterial DNA extraction: One ml of cultured cells was transferred into a 1.5 microtube, then centrifuged at 14000 xg for 1 min and the supernatant was discarded. The cell pellets were re-suspended in 300 µl of Tris (pH 7.5), EDTA and RNase. Then 2 µl aliquots of lysozyme solution were added and incubated at 37ºC for 60 min, the tubes were then centrifuged at 14000 g for 1 min and the supernatants were discarded. The pellets were re-suspended in 300 µl of cell lysis solution; SDS (sodium dodecyl sulfate), Tritone X100 which dissolves the phospholipids and protein components of the cell membrane.

Add 1.5 µl of RNase solution and incubate at 37ºC for 45 min and cool on ice for 1 min. Add 100 µl of protein precipitation solution (Guanidinum isothiocyanate) and vortex vigorously for 20 sec then centrifuge at 14000 g for 5 min. Transfer the supernatant to a clean 1.5 ml microtube containing 300 µl isopropanol 99% then centrifuge at 14000 g

for 1 min. Discard the supernatant then add 500 µl ethanol 80% and centrifuge at 15000 g for 1 min. Discard the supernatant and air dry at room temperature for 15 min. Add 100 µl of DNA hydration solution to the dried DNA pellet. Hydrate the DNA by incubating at 65ºC for 1 hr then store the DNA at 4ºC. The sequences of *icaA* and *icaD* were taken from the Gene Bank sequence database of the National Center for Biotechnology Information. Primers specific for *icaA* and *icaD* were picked on the gene sequence by the primer 3 program. For the detection of *icaA*, 5´- TCTCTTGCAGGAGCAATCAA was used as forward primer and 5´- TCAGGCACTAACATC CAGCA was used as a reverse primer. The two primers include a 188-bp region. For detection of *icaD*, 5´- ATGGTCAAGCCCAGACAGAG was used as a forward primer and a 5´- CGTGTTT TCAACATTTAATGC was used as reverse primer. The two primers include a 198-bp region. PCR was performed in a DNA thermal cycler. The reaction volume was 25 µl containing 2.5 µl of each the forward and reverse primers, together with 5 µl of the extracted DNA, 10 µl of Master Mix and 5 µl of distilled water. Thermal step program for both *icaA* and *icaD* genes include the following incubation at 94ºC for 5 min, followed by 35 cycles at 94ºC for 30 sec (denaturation), 56ºC for 30 sec (annealing), 72ºC for 30 sec (extension), and 72ºC for 1 min.

Ten µl of the PCR mixture was analyzed by agarose gel electrophoresis (1.5% agarose in Tris borate–EDTA stained with 1 µl Ethidium bromide 5% w/v). The gene ruler 100 bp DNA ladder was used as a DNA size marker.

Detection of bap protein by polyacrylamide gel electrophoresis (SDS-PAGE)

Procedures and preparation of solutions and reagents were carried out according to the previous method[19], as follow:

SDS-PAGE solutions

Stock 1

Acrylamide (30 g) and 0.8 g bis N,N methylene bisacrylamide were dissolved in 100 ml distilled water. The solution was then filtered through glass filter under vacuum and kept at 4 °C in a dark bottle.

Stock 2

Tris-HCl (18.2 g) was dissolved in 50 ml distilled water, pH was adjusted with HCl to 8.8 and the volume was then completed to 100 ml distilled water and the solution was stored in the refrigerator.

Stock 3

Tris-HCl (6 g) dissolved in 50 ml distilled water. The pH was adjusted with HCl to 6.8 and the volume was then completed to 100 ml distilled water and the solution was stored in the refrigerator.

Protein analysis

Preparation of samples

Strains were grown overnight in TSB at 30°C. Following centrifugation of 1 ml of culture, cells were harvested, washed, and finally suspended in 75 µl of PBS buffer containing lysostaphin (12.5 µg/ml; Sigma). After 2 hrs of incubation at 37°C, equal volume of Laemmli buffer was added and boiled for 10 min. After centrifugation, 20 µl of the supernatant was used for SDS-PAGE (12% separating gel, 4.5% stacking gel) and proteins were stained with Coomassie brilliant blue R250 (0.25%; Sigma).

Laemmli buffer preparation: Basic 2X Laemmli buffer prepared by 4% SDS, 20% glycerol, 10% 2- mercaptoethanol, 0.004% bromophenol blue and 0.125 M TrisHCl.

Sample loading and application: After gel stacking, comb removed, wells washed with distilled water to remove not polymerized acrylamide and straighten the teeth of the wells. One hundred µl of each sample was loaded into the bottom of the wells, using a micro liter syringe.

Electrophoresis

The molded gel was completed with electrode. Electrophoresis was carried out at 140V for 120 min. About 0.5g Coomassie R-250 brilliant blue was dissolved in 250 ml methanol, 50 ml glacial acetic acid, completed by distilled water to 500 ml, mixed and kept at room temperature. Gels were stained overnight in 200 ml of Coomassie brilliant blue R-250 solution. De-staining of protein was performed in 200 ml of de-staining solution which composed of 250 ml methanol, 50 ml glacial acetic acid and 200 ml distilled water with gentle shaking. The de-staining solution was changed several times until background color was removed. The Lab Image (2006) program was used in molecular weight determination of protein

RESULTS

Two hundred fifty Staphylococcal strains

were isolated from 520 different clinical samples. One hundred sixty were CPS and 90 were CNS (Table 1).

Table 1. Samples and numbers of CPS and CNS

Clinical Samples	CPS	CNS	Total strains
Wound Swab	55	15	70
Sputum	42	18	60
Throat swab	18	12	30
Ear discharge	12	5	17
Urine	23	35	58
Blood	10	5	15
Total	160	90	250

Ability of Staphylococcal strains to form biofilm:

The results revealed that both CPS and CNS isolated from different clinical samples were able to produce biofilm by the three used methods. CRA method revealed that 82.5% (132/160) of CPS strains were biofilm producers while by using ST method, the results were 64.3% (103/160). For CNS, the percentages were 71.1% (64/90) and 55.5% (50/90) using CRA and ST respectively. Screening by TCP revealed that 85% (136/160) of CPS strains and 84% (76/90) of CNS were biofilm producers (Tables 2, 3 and 4 and Fig. 1 and 2).

PCR detection of *icaA* and *icaD* genes

All biofilm producing strains isolated from different sources were found to be positive for both *icaA* and *icaD* genes, giving a 188-bp band for *icaA*, and a 198-bp band for *icaD*. All

Table 2. Biofilm production by Staphylococci using CRA method

Specimens	Wound Swab		Sputum		Throat swab		Ear discharge		Urine		Blood		No**
Staphylococcal strains	N	No*	N	No*	N	No*	N	No*	N	No*	N	No*	
CPS	55	46	42	37	18	14	12	9	23	18	10	8	132
CNS	15	10	18	11	12	9	5	3	35	28	5	3	64
Total	70	56	60	48	30	23	17	12	58	46	15	11	196

No**= Total number of biofilm-producing strains for the corresponding sample.
No*= Number of biofilm-producing strains for the corresponding sample.
N = Number of strains isolated from the corresponding sample.

Table 3. Biofilm production by Staphylococci using ST method

Specimens	Wound Swab		Sputum		Throat swab		Ear discharge		Urine		Blood		No**
Staphylococcal strains	N	No*	N	No*	N	No*	N	No*	N	No*	N	No*	
CPS	55	34	42	30	18	10	12	7	23	15	10	7	103
CNS	15	9	18	8	12	7	5	2	35	22	5	2	50
Total	70	43	60	38	30	17	17	9	58	37	15	9	153

No**= Total number of biofilm-producing strains for the corresponding sample.
No*= Number of biofilm-producing strains for the corresponding sample.
N = Number of strains isolated from the corresponding sample.

Table 4. Biofilm production by Staphylococci using TCP method

Specimens	Wound Swab		Sputum		Throat swab		Ear discharge		Urine		Blood		No**
Staphylococcal strains	N	No*	N	No*	N	No*	N	No*	N	No*	N	No*	
CPS	55	47	42	37	18	14	12	9	23	21	10	8	136
CNS	15	13	18	14	12	11	5	3	35	31	5	4	76
Total	70	60	60	51	30	25	17	12	58	52	15	12	212

No**= Total number of biofilm-producing strains for the corresponding sample.
No*= Number of biofilm-producing strains for the corresponding sample.
N = Number of strains isolated from the corresponding sample.

Table 5. Effect of 1% glucose on biofilm production by TCP method

Specimens	Wound Swab		Sputum		Throat swab		Ear discharge		Urine		Blood		No**
Staphylococcal strains	N	No*	N	No*	N	No*	N	No*	N	No*	N	No*	
CPS	55	50	42	39	18	16	12	10	23	22	10	9	146
CNS	15	14	18	16	12	11	5	4	35	32	5	4	81
Total	70	64	60	55	30	27	17	14	58	54	15	13	227

No**= Total number of biofilm-producing strains for the corresponding sample.
No*= Number of biofilm-producing strains for the corresponding sample.
N = Number of strains isolated from the corresponding sample.

Table 6. Effect of 4 % NaCl on biofilm production by TCP method

Specimens	Wound Swab		Sputum		Throat swab		Ear discharge		Urine		Blood		No**
Staphylococcal strains	N	No*	N	No*	N	No*	N	No*	N	No*	N	No*	
CPS	55	22	42	19	18	10	12	5	23	8	10	4	68
CNS	15	5	18	9	12	5	5	1	35	15	5	1	36
Total	70	27	60	28	30	15	17	6	58	23	15	5	104

No**= Total number of biofilm-producing strains for the corresponding sample.
No*= Number of biofilm-producing strains for the corresponding sample.
N = Number of strains isolated from the corresponding sample.

Table 7. Effect of pH 7.7 on biofilm production by TCP method

Specimens	Wound Swab		Sputum		Throat swab		Ear discharge		Urine		Blood		No**
Staphylococcal strains	N	No*	N	No*	N	No*	N	No*	N	No*	N	No*	
CPS	55	27	42	21	18	14	12	7	23	12	10	5	86
CNS	15	6	18	11	12	6	5	2	35	19	5	2	46
Total	70	33	60	33	30	20	17	9	58	31	15	7	133

No**= Total number of biofilm-producing strains for the corresponding sample.
No*= Number of biofilm-producing strains for the corresponding sample.
N = Number of strains isolated from the corresponding sample.

Table 8. Effect of pH 6.5 on biofilm production by TCP method:

Specimens	Wound Swab		Sputum		Throat swab		Ear discharge		Urine		Blood		No**
Staphylococcal strains	N	No*	N	No*	N	No*	N	No*	N	No*	N	No*	
CPS	55	24	42	20	18	13	12	6	23	10	10	4	77
CNS	15	4	18	10	12	4	5	2	35	15	5	1	36
Total	70	28	60	30	30	17	17	8	58	25	15	5	113

No**= Total number of biofilm-producing strains for the corresponding sample.
No*= Number of biofilm-producing strains for the corresponding sample.
N = Number of strains isolated from the corresponding sample.

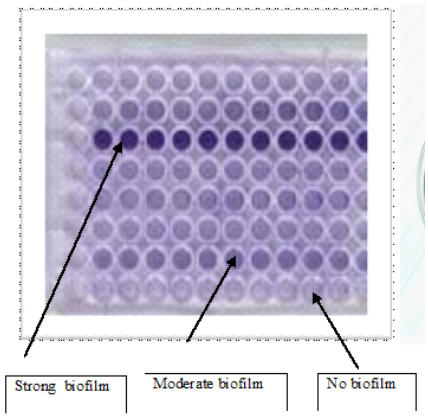


Fig. 1. Biofilm formation by TCP method

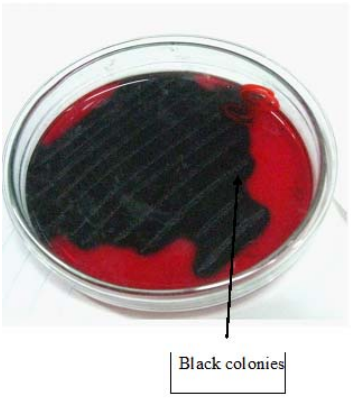
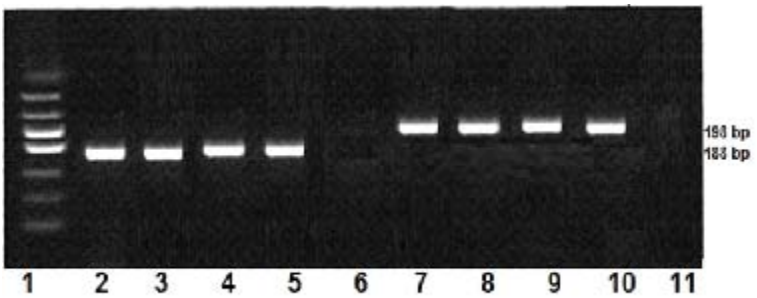
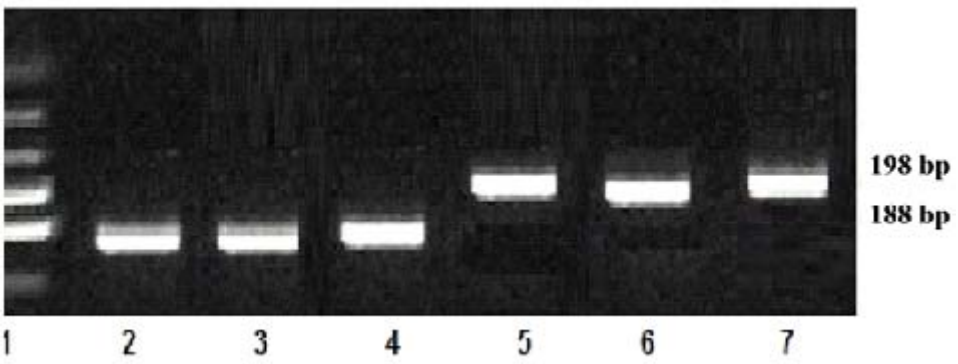


Fig. 2. Biofilm formation by CRA method.



Lane (1): marker 100 bp.
Lanes (7), (8), (9) and (10): 198 bp of icaD for CPS.
Lanes (2), (3), (4) and (5): 188 bp of icaAfor CPS.
Lanes (6) and (11): no band of non biofilm forming CPS.

Fig. 3. Detection of *icaA and icaD* genes for CPS



Lane (1): marker 100 bp. Lane (2), (3) and (4): 188 bp of icaAfor CNS. Lane (5), (6) and (7): 198 bp of icaD for CNS.

Fig.4. Detection of *icaA and icaD* genes for CNS

strains which were positive for *icaA* were positive for *icaD* (Fig. 3 and 4).

Biofilm-associated protein (Bap) expression

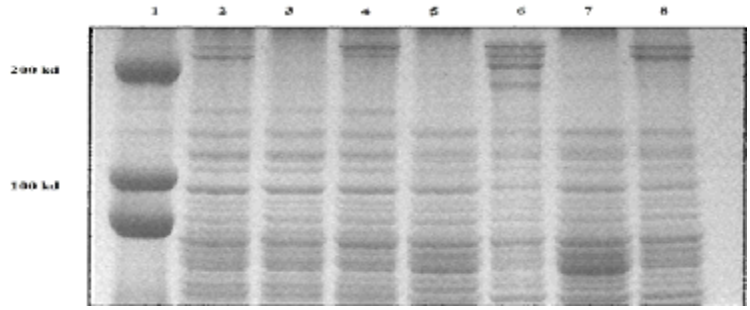
A double band that migrated at a position corresponding to 230 and 240 kDa was detected by SDS-PAGE of the total protein extract from the isolated strains. Analysis of the capacity to form a biofilm (on a polystyrene surface after 24 hrs) demonstrated that biofilm forming strains express Bap (Fig. 5).

DISCUSSION

Biofilm formation is a process in which bacteria undergo switch from a unicellular state to a multicellular state where subsequent growth results in structured communities and cellular differentiation[20]. The majority of human infections are caused by biofilms. The biofilm as a mode of growth enhances the pathogenicity of *Staphylococcus spp*. considerably, because once they adhere, Staphylococci embed themselves in a protective, self-produced matrix of extracellular polymeric substances (EPSs)[21].

In our study, 250 Staphylococcal strains isolated from different clinical samples were included in this study (160CPS and 90CNS). Biofilm formation was studied using TCP, ST, and CRA method. Out of the 160 CPS, 136 (85%) were biofilm producer by TCP. Out of the 90 CNS, 76 (84%) were biofilm producer using the same method. Screening by CRA showed that 132 strains (69.5%) were biofilm producers for CPS and 64 (71.1%)were biofilm producers for CNS. Screening on ST method showed that 103 (54.2%) for CPS and 50 (55.5%) were biofilm producer for CNS. These results are higher than those obtained by Yazdani *et al*[22], whore ported that 27 (54%) of 50 *S.aureus* strains were slime-producing and 23 (46%)of 50 strains produced smooth and red colonies when tested by CRA method. Quantitative biofilm production was determined by microtiter plate assay; fifty strains were identified, of them 26 (52%) were positive biofilm forming, and 24(48%) isolates were negative. Two strains produced black colony on CRA plate, were biofilm negative on microtiter plate. The difference in the results with others may be attributed to variance in the regional environmental conditions. However, the results are, to some extent, close to those obtained by Sheila *et al*., 2010[23], who reported that biofilm production by CRA, Micro Plate (MP), and ST tests in CPS and CNS strains were 77.7%, 74.4%; 66.6%, 44.4%, and 36.6%, 34.4%, respectively. While these results are similar to those obtained by Carla-Renata *et al*.[24], who reported that biofilm production by CRA and MP tests in CNS strains; Out of 80 *S. epidermidis* strains isolated from orthopedic implant infections 57% were positive for CRA test. Differently, by the MP method, 66% of the strains were found to be biofilm-producer. In the previous studies[16,17,18], they studied 91 Staphylococcal strains (68 *S. epidermidis* and 23 *S.aureus*) isolated from intravenous catheters. They found that 48.5% (30 of 68) of *S. epidermidis* and 60.8% (14 of 23) of *S. aureus* isolates were biofilm producers. While these results are nearly similar to those obtained by Mathura *et al*.[25] who reported that 88(57.8%) of 152 Staphylococcal isolates displayed a biofilm-positive phenotype under the optimized conditions of MP method. These strains were classified as high biofilm producers, 22 (14.47 %)and moderate biofilm producers, 60 (39.4 %) while in 70 isolates (46.0 %) weak or no biofilm was detected. Weak producers were difficult to discriminate from biofilm negative isolates. In our study, the effect of physiological changes was tested. Addition of 1% glucose increased biofilm formation for CPS and CNS strains and as the concentration of glucose increases the biofilm production increases (Table 5). Whereas addition of 4% NaCl decreased biofilm formation for both CPS and CNS strains (Table 6). These results are closely related to the previous studies[6,26].

Kati *et al*.[27] found that biofilm formation was shown to be promoted by increasing concentrations of glucose. Without glucose



Lane (1): Protein Marker. Lane (2), (4), (6) and (8): *S.epidermidis*. Lane (3), (5) and (7): *S.aureus*

Fig.5. Bap expression

supplementation, no significant biofilm formation (A530, <0.5) was observed. Interestingly, supplementation with 0.2% glucose was sufficient to induce a visible biofilm (A530, >1.5) and a further increase in glucose concentration up to 1% slightly increased biofilm formation (Table 5). The previous studies were reported that supplementation of growth media with different sugars increased biofilm formation significantly[28,29]. It is noticeable that biofilm formation depends on basic media. These results indicate a strong dependence between biofilm formation in *S. aureus* and the environmental conditions of growth, which seem to be even more pronounced than in *S. epidermidis*. This indicates agreement with our results (Table7), where slight low pH decreased biofilm formation (table 8). Johannes *et al*.,[30] evaluated a tissue culture plate assay and standard tube test, as well as CRA, using the two basic media trypticase soy broth and brain heart infusion broth with different sugar supplements for detection of biofilm formation in 128 *ica*-positive *S. aureus* isolates. Of the *S. aureus* strains, 57.1% displayed a biofilm-positive phenotype under optimized conditions in the TCP test[6].

In our study biofilm and non biofilm producing Staphylococci strains were subjected to PCR for determining *icaA* and *icaD* genes to identify and confirm biofilm producing strains. It was found that all biofilm producing Staphylococci strains were positive for *icaA* and *icaD* genes. These results correlate well with those reported previously[6,17,18,19]. They found that all Staphylococcal biofilm positive strains were positive for *icaA* and *icaD* genes which are required for slime synthesis. In addition, our results showed that both genes (*icaA*and *icaD*) were present in all biofilm producing strains, indicating the important role of *ica* genes as virulence markers in Staphylococcal infections. In the previous studies[32,33], they detected *ica* genes in all *S. aureus* isolates by PCR. In another study[30], they observed that differential results were obtained in respect of the incidence of *icaADBC* in *S. aureus* by PCR. Gad *et al.*[15] reported that out of 18 *S. aureus* strains, 15 (83.3%) were biofilm producers and out of 35 *S. epidermidis* strains, 31 (88.6%) were biofilm producers. Staphylococcal strains were further classified as high (56.6%), moderate (30.2%) and non biofilm producers (13.2%). All biofilm producing strains were positive for *icaA*and *icaD* genes, and all biofilm negative strains were negative for both genes. While our results differ than those obtained by Yazdani *et al*.[22] who reported that, only 54% of the strains produced biofilm in vitro, but all isolates demonstrated *icaAD* gene.

This study showed that bap expression allows and enhances biofilm formation. A double band at a position corresponding to 230 and 240 kDa was detected by SDS-PAGE of the total protein extract. These results are closely related to those obtained by Cucarella *et al*.[33] and Abdi-Ali *et al*.[34], who stated that the implication of biofilm in bacterial infection in many species has triggered an increasing interest in the characterization of genes involved in biofilm formation. The bap gene is a new identified gene that encodes the biofilm-associated protein, BAP, which is involved in biofilm formation in *S.aureus*. Finally, this study indicated that *icaA* and *icaD* genes play key roles in the process for biofilm formation of Staphylococci and this is similar to that obtained by Speziale *et al*.[8].

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