A Microtiter plate assay for *Staphylococcus aureus* biofilm quantification at various pH levels and hydrogen peroxide supplementation

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SUMMARY

Biofilm formation is the leading cause of the pathogenesis of *S. aureus* associated with biomaterial infections. In *S. aureus* polysaccharide intercellular adhesin (PIA) was encoded by *ica*A and *ica*D genes. Production of PIA is currently responsible for staphylococcal biofilm development. In this study, *S. aureus* strains isolated from auricular infection (n = 46) and *S. aureus* ATCC 25923 were phenotyped and genotyped. Slime production was assessed using Congo red agar plate assay. In order to determine the biofilm formation capacity at various pH levels of the studied *S. aureus* strains, microtiter plate assay was performed. The strains were grown in medium adjusted at various pH levels (3, 5, 7, 9 and 12) and medium supplemented with hydrogen peroxide 3% (v/v). Qualitative biofilm production of *S. aureus* revealed that 56.5% of strains were slime producers. In addition 78.26% of strains were *ica*A and *ica*D positive. Quantitative biofilm showed that biofilm production depended on the pH value of the medium. At highly acidic (pH 3) and alkaline (pH 12) levels, biofilm formation was lower, while at pH 7 the adhesion was moderate. In addition, the cells adhered weakly after 3% hydrogen peroxide supplementation. Our results suggest that pH was a stress factor that led some *S. aureus* strains to produce the biofilm.

KEY WORDS: Staphylococcus aureus, Slime production, Biofilm, Hydrogen peroxide, *ica* gene, pH

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INTRODUCTION

Staphylococcus aureus is the first bacterium implicated in nosocomial infections and is one of the most important pathogens in foreign body-related infections. In addition, *S. aureus* has the capacity to adhere to catheters and other indwelling medical devices and form biofilm on polymeric surfaces (Cramton *et al.,* 1999; Fowler *et al.,* 2001). This ability depends on the production of polysaccharide intercellular adhesin (PIA) encoded by the intercellular adhesion gene cluster *ica*ADBC (Cramton *et al.,* 1999, Mckenney *et al.,* 1999). The polysaccharide intercellular adhesin is composed of linear β-1,6-linked glucosaminylglycans. It is synthesized in vitro from UDP-*N*-acetylglucosamine by the enzyme *N*-acetylglucosaminyltranferase, which is encoded by the intercellular adhesion (*ica*) locus, in particular by the *icaA* gene. Sole expression of *ica*A induces only low enzymatic activity, but coexpression of *ica*A with *ica*D leads to a significant increase in activity and is related to phenotypic expression of the capsular polysaccharide (Gerke *et al.,* 1998). The initial attachment of staphylococcal cells on a biomaterial is followed by bacterial accumulation and formation of a mature biofilm (Götz, 2002). Established biofilms can tolerate antimicrobial agents, and are extraordinarily resistant to phagocytosis, making biofilms extremely difficult to eradicate (Lewis, 2001). Biofilm formation on the implant surface is believed to take place in at least two stages: primary reversible adsorption (Marshall *et al.,* 1976), ir-

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reversible adhesion of microorganisms to the device and the formation of stable biofilm matrix over surface of the implant (Characklis, 1981). The rates of these processes vary widely depending on the environmental conditions and the type of microorganisms. Moreover, the characteristics of the suspending medium, such as pH, ionic strength and temperature, are also considered as important factors in altering the physicochemical properties of a bacterial surface and consequently the microbial adhesion to the substrate (Hamadi *et al.,* 2004). The effect of pH levels of the suspending medium has received comparatively little attention. All these studies found that the density of adhering cells increases when the pH of the medium was near to the isoelectric point of the substrate surface (Mozes *et al.,* 1987; Webb *et al.,* 1999).

Hydrogen peroxide is a powerful oxidizing agent, easily handled and applied on non-critical items. It is used in the disinfection of medical and dental devices in health care routine (Pitts *et al.,* 2003). Hydrogen peroxide (H_2O_2) has been used as a wound disinfectant for a long time (Tschesche *et al.,* 1951), and effective on Gram negative and Gram positive bacteria as well as yeasts (Yosphe-Purer & Eylan 1968). In hospital, acidic and alkalinic pH-based disinfectants are frequently used in cleaning of medical instruments and surfaces.

The aim of this study was to assess the effect of pH levels (3, 5, 7, 9, and 12) and the efficacy of hydrogen peroxide supplementation on *Staphylococcus aureus* biofilm formation.

MATERIALS AND METHODS

Bacterial isolates

Forty-six *S. aureus* strains were collected over a 10 month period from patients with an auricular infection. Sampling was performed using sterile swabs. Pus was directly plated on sheep blood agar plates containing 4% NaCl. Suspected colonies of *Staphylococcus aureus* were confirmed by their positive Gram stain, catalase positive and DNAse positive reaction (Oxoid, Basingstoke, UK) and the presence of free plasma coagulase using rabbit plasma (Bio-Merieux, France). Biochemical identification was performed using API ID32 Staph strips (Bio-Merieux, France) according to the manufacturer's recommendation and the results were read using an automated microbiological mini-API (Bio-Merieux, France).

Phenotypic characterization of bacteria-producing slime

Qualitative detection of slime producer strains was studied by culturing the bacteria on Congo red agar plate (CRA) made by mixing 36 g saccharose (Sigma Chemical Company, St. Louis, MO) with 0.8 g Congo red in one litre of Brain heart infusion agar (Biorad, USA) as previously described (Freeman *et al.,* 1989; Zmantar *et al.,* 2006, Snoussi *et al.,* 2008). The strains were incubated at 37°C for 24 h under aerobic conditions. Results were interpreted as follows: Very black, black and almost black colonies, were considered to be normal slime-producing strains, while very red, red and bordeaux colonies were classified as non-slime-producing strains (Arciola *et al.,* 2002; Ziebuhr *et al.,* 1999).

Detection of *ica*A and *ica*D loci

Staphylococcus aureus strains were grown overnight at 37°C on Sheep blood agar plates. One colony was suspended in 1 ml LB broth (Sigma Chemical Company, St. Louis, MO) at 37°C for 24 h. Chromosomal DNA was extracted using a Wizard Genomic Purification Kit (Promega, Lyon, France) and adjusted at 50 ng/µl using Ultraspec 2100 pro (Amersham Biosciences Europe GmbH, France). The presence of *ica*A and *ica*D genes was detected by polymerase chain reaction (PCR) using forward and reverse primers for *ica*A and *ica*D, as described previously (Montanaro *et al.,* 1999). A PCR mixture (25 µl) contained 1 mM forward and reverse primers, dNTP mix (100 mM each of dATP, dCTP, dGTP and dTTP), 1 U of GO *Taq* DNA polymerase (Promega), 5 µl green Go *Taq* buffer (5X), and DNA template (50 ng). PCR conditions included initial denaturation (94°C for 5 min), followed by 30 cycles of denaturation (94°C for 30s), annealing (55°C for 30s), and extension (72°C for 45s), followed at the end of cycling with final extension (72°C for 10 min). PCR products (5µl) were analysed on 2% (wt/v) agarose gel stained with ethidium bromide (0.5 µg/µl), and visualized under ultraviolet transillumination and photographed using Gel Doc XR apparatus (Biorad, USA).

Quantitative biofilm production assay by *S. aureus* cells as a function of pH levels and hydrogen peroxide

Biofilm production by *S. aureus* strains grown in trypticase soy broth (TSB) was determined using a semi-quantitative adherence assay on 96-well tissue culture plates (Nunc, Roskilde, Denmark) as described previously (Christensen *et al.,* 1985; Mack *et al.,* 2001, Knobloch *et al.,* 2001). After incubation for 24 h at 37°C, the absorbance at 570 nm (OD_{570}) was recorded as a measure of total growth. An overnight culture grown in TSB (Biorad) at 37°C was diluted to 1:100 in TSB with 2% (w/v) glucose to maximize *ica* operon induction, as reported elsewhere (Rachid *et al.,* 2000). A total of 200 µl of these cell suspensions was transferred in a U-bottomed 96-well microtiter plate. Each strain was tested in triplicate. Wells with sterile TSB alone was served as controls. *S. aureus* ATCC 25923 was used as the positive control.

The plates were incubated aerobically for 24 h at 37°C. Furthermore, the culture was removed and plates were washed three times with 200 µl of phosphate-buffered saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl at pH 7.4) to remove non adherent cells and dried in an inverted position. Adherent biofilm was fixed with 95% ethanol and was stained with 100µl of 1% (wt/vol) crystal violet (Merck) for 5 min. Then, unbound crystal violet was removed and the wells were washed three times with 300 µl of sterile distilled water. The water was then cleared and the microtiter plate was air dried for 2 h. The optical density (OD) of each well was measured at 570 nm using an automated Multiskan reader (GIO. De Vitae, Rome, Italy). Biofilm formation was categorized as highly positive ($OD_{570} \geq 1$), low-grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$). To analyze the effect of pH and hydrogen peroxide on the degree of biofilm formation, the pH of the medium was adjusted (3, 5, 7, 9 and 12) and the TSB was supplemented with 3% (v/v) of hydrogen peroxide (Riedel-deHaën, Germany).

Increased biofilm formation caused by the effect of pH and hydrogen peroxide supplementation was defined as being at least $OD_{570} = 0.2$ when the strain was primary-negative, or at least double OD_{570} when the strain was low-grade positive, as previously described (Knobloch *et al.,* 2001).

Statistical analysis

Each analysis was performed using the S.P.S.S. 13.0 statistics package for Windows. The differences in the degree of biofilm formation were examined by the Friedman test, followed by the Wilcoxon signed ranks test. *P*-values of <0.05 were considered significant.

RESULTS

Phenotypic determination of slime production of *Staphylococcus aureus* strains

The slime production of each strain was assessed by means of cultures on CRA plates (Table 1) as described previously (Freeman *et al.,* 1989). A total of 46 *S. aureus* strains were isolated from auricular infection and were investigated for slime production. Results were interpreted as described elsewhere (Ziebuhr *et al.,* 1999; Arciola *et al.,* 2002). Among the forty-six clinical *S. aureus* isolates, 26 strains (56.5%) were slime producers developing almost black or very black colonies on CRA plate and the remaining 20 strains were non-producers developing red or bordeaux colonies (Table 1).

Detection of *ica*A and *ica*D loci by PCR

The PCR technique was applied to the 46 *Staphylococcus aureus* strains as presented in Table 1. We noted in this study that strains positive for *ica*A were also positive for *ica*D, giving a 188-bp band for the *ica*A gene and a 198-bp band for the *ica*D. In addition among the 46 tested strains, 36 (78.26%) were positive for *ica*A and *ica*D.

Relationships between the *ica* operon and slime production

In this study, ten *ica*A- and *ica*D- strains were unable to produce biofilm on CRA, giving smooth, red or Bordeaux colonies. Furthermore, among the 36 *ica*A and *ica*D positive strains, 26 were slime producers forming black colonies by the CRA method, and ten were slime negative (Table 1).

Biofilm formation by *S. aureus* at various pH levels

Biofilm formation of *S. aureus* strains was evaluated in 96 wells plate with TSB at different pH levels (3, 5, 7, 9 and 12). *S. aureus* ATCC 25923 was used as reference strains.

Table 2 presents the results of the OD_{570} at the different pH values and H_2O_2 supplementation. We noted that at pH 7, one strain (Sa1) was classified as highly biofilm-positive ($OD_{570} \geq 1$). In addition, 28 strains showed low-grade biofilm formation ($0.1 \geq OD_{570} < 1$), and 17 were biofilm-negative (Table 1). However Sa1 was *ica*A and *ica*D positive and it was unable to produce slime on

TABLE 1 - *Relationships between presence of icaA, icaD, slime production and *OD_{570} in clinical Staphylococcus aureus*

Sample	*icaA/icaD*	*Biofilm phenotype on (CRA)*	*Slime Production*	**OD_{570}*
ATCC	icaA+/icaD+	black	Producer	++
Sa1	icaA+/*icaD*+	red	Non producer	++++
Sa2	icaA+/*icaD*+	very black	Producer	++
Sa3	icaA+/*icaD*+	almost black	Producer	++
Sa4	icaA+/*icaD*+	black	Producer	++
Sa5	icaA+/*icaD*+	almost black	Producer	++
Sa6	icaA+/*icaD*+	very black	Producer	++
Sa7	icaA+/*icaD*+	very black	Producer	++
Sa8	icaA+/*icaD*+	bordeaux	Non producer	-
Sa9	icaA+/*icaD*+	very black	Producer	++
Sa10	icaA+/*icaD*+	bordeaux	Non producer	++
Sa11	icaA+/*icaD*+	black	Producer	-
Sa12	icaA+/*icaD*+	black	Producer	++
Sa13	icaA+/*icaD*+	black	Producer	++
Sa14	icaA+/*icaD*+	almost black	Producer	++
Sa15	icaA+/*icaD*+	black	Producer	++
Sa16	icaA+/*icaD*+	black	Producer	-
Sa17	icaA+/*icaD*+	black	Producer	++
Sa18	icaA+/*icaD*+	black	Producer	++
Sa19	icaA+/*icaD*+	almost black	Producer	++
Sa20	icaA+/*icaD*+	black	Producer	++
Sa21	icaA+/*icaD*+	black	Producer	++
Sa22	icaA+/*icaD*+	black	Producer	++
Sa23	icaA+/*icaD*+	almost black	Producer	++
Sa24	icaA+/*icaD*+	very black	Producer	-
Sa25	icaA+/*icaD*+	black	Producer	++
Sa26	icaA+/*icaD*+	very black	Producer	++
Sa27	icaA+/*icaD*+	bordeaux	Non producer	++
Sa28	icaA+/*icaD*+	red	Non producer	++
Sa29	icaA+/*icaD*+	red	Non producer	++
Sa30	icaA+/*icaD*+	almost black	Producer	++
Sa31	icaA+/*icaD*+	red	Non producer	-
Sa32	icaA+/*icaD*+	almost black	Producer	-
Sa33	icaA+/*icaD*+	red	Non producer	-
Sa34	icaA+/*icaD*+	almost black	Producer	-
Sa35	icaA+/*icaD*+	bordeaux	Non producer	++
Sa36	icaA+/*icaD*+	red	Non producer	-
Sa37	*icaA-/icaD-*	bordeaux	Non producer	-
Sa38	*icaA-/icaD-*	bordeaux	Non producer	++
Sa39	*icaA-/icaD-*	red	Non producer	++
Sa40	*icaA-/icaD-*	bordeaux	Non producer	-
Sa41	*icaA-/icaD-*	red	Non producer	-
Sa42	*icaA-/icaD-*	bordeaux	Non producer	-
Sa43	*icaA-/icaD-*	red	Non producer	++
Sa44	*icaA-/icaD-*	bordeaux	Non producer	++
Sa45	*icaA-/icaD-*	bordeaux	Non producer	-
Sa46	*icaA-/icaD-*	bordeaux	Non producer	-

*Strongly biofilm positive (++++), low grade biofilm-positive (++) and biofilm negative (-).

CRA (Table 1). Furthermore, among the 28 low-grade biofilm strains, 24 harboured the *ica*A and *ica*D genes.

We also noted that among the low-grade biofilm strains, seven isolates were biofilm negative on CRA plate, forming red or bordeaux colonies. Among the biofilm negative strains in microtiter plate (OD_{570} <0.1), 11 out of 17 strains were *ica*A

TABLE 2 - *Biofilm formation by Staphylococcus aureus at different pH value and H_2O_2*

Strains	*pH3*		*pH5*		*pH7*		*pH9*		*pH12*		H_2O_2	
	OD_{570}	SD[a]	OD_{570}	SD	OD_{570}	SD	OD_{570}	SD	OD_{570}	SD	OD_{570}	SD
ATCC	0.006	±0.004	0.382	±0.040	0.495	±0.100	0.440	±0.052	0.013	±0.004	0.293	±0.058
Sa1	0.026	±0.006	0.073	±0.020	1.024	±0.059	0.110	±0.002	0.034	±0.028	0.117	±0.017
Sa2	0.034	±0.0004	0.121	±0.056	0.290	±0.116	0.096	±0.025	0.051	±0.009	0.056	±0.011
Sa3	0.019	±0.006	0.100	±0.027	0.210	±0.067	0.114	±0.013	0.057	±0.023	0.193	±0.136
Sa4	0.012	±0.005	0.020	±0.005	0.355	±0.142	0.079	±0.044	0.023	±0.010	0.030	±0.007
Sa5	0.008	±0.004	0.054	±0.002	0.275	±0.198	0.172	±0.018	0.017	±0.015	0.054	±0.004
Sa6	0.017	±0.004	0.039	±0.009	0.317	±0.167	0.121	±0.082	0.019	±0.006	0.108	±0.018
Sa7	0.031	±0.020	0.088	±0.010	0.250	±0.125	0.058	±0.012	0.027	±0.014	0.056	±0.015
Sa8	0.018	±0.002	0.007	±0.001	0.012	±0.009	0.019	±0.008	0.020	±0.003	0.024	±0.005
Sa9	0.016	±0.007	0.054	±0.010	0.146	±0.023	0.067	±0.024	0.084	±0.009	0.024	±0.004
Sa10	0.015	±0.002	0.117	±0.031	0.324	±0.186	0.073	±0.022	0.032	±0.009	0.048	±0.012
Sa11	0.019	±0.002	0.040	±0.009	0.090	±0.013	0.100	±0.020	0.031	±0.010	0.122	±0.019
Sa12	0.017	±0.002	0.061	±0.030	0.302	±0.118	0.104	±0.023	0.039	±0.014	0.035	±0.010
Sa13	0,019	±0.003	0.415	±0.027	0.160	±0.009	0.076	±0.005	0.013	±0.005	0.056	±0.004
Sa14	0.018	±0.006	0.228	±0.084	0.259	±0.128	0.227	±0.099	0.016	±0.008	0.220	±0.031
Sa15	0.015	±0.005	0.235	±0.036	0.306	±0.123	0.056	±0.021	0.020	±0.005	0.075	±0.020
Sa16	0.032	±0.015	0.093	±0.059	0.096	±0.028	0.034	±0.009	0.025	±0.004	0.044	±0.008
Sa17	0.021	±0.006	0.101	±0.012	0.127	±0.052	0.143	±0.024	0.017	±0.002	0.061	±0.007
Sa18	0.018	±0.003	0.114	±0.027	0.063	±0.023	0.117	±0.018	0.015	±0.001	0.045	±0.006
Sa19	0.016	±0.005	0.106	±0.045	0.205	±0.073	0.049	±0.021	0.032	±0.019	0.065	±0.019
Sa20	0.016	±0.001	0.068	±0.021	0.117	±0.032	0.179	±0.007	0.019	±0.008	0.054	±0.001
Sa21	0.025	±0.001	0.090	±0.057	0.179	±0.029	0.358	±0.073	0.027	±0.010	0.121	±0.014
Sa22	0.031	±0.002	0.440	±0.054	0.148	±0.031	0.214	±0.084	0.032	±0.006	0.128	±0.029
Sa23	0.019	±0.002	0.135	±0.023	0.222	±0.125	0.269	±0.056	0.021	±0.009	0.034	±0.006
Sa24	0.023	±0.004	0.063	±0.024	0.084	±0.041	0.058	±0.009	0.021	±0.009	0.091	±0.045
Sa25	0.016	±0.003	0.099	±0.028	0.277	±0.076	0.122	±0.051	0.012	±0.002	0.133	±0.026
Sa26	0.018	±0.002	0.356	±0.062	0.179	±0.093	0.337	±0.034	0.031	±0.001	0.050	±0.003
Sa27	0.008	±0.004	0.146	±0.001	0.129	±0.005	0.069	±0.015	0.072	±0.049	0.038	±0.009
Sa28	0.020	±0.003	0.098	±0.016	0.306	±0.221	0.073	±0.022	0.962	±0.667	0.160	±0.030
Sa29	0.032	±0.015	0.172	±0.028	0.060	±0.007	0.067	±0.025	0.022	±0.006	0.115	±0.022
Sa30	0.018	±0.002	0.238	±0.170	0.125	±0.038	0.042	±0.011	0.015	±0.005	0.194	±0.076
Sa31	0.018	±0.008	0.087	±0.013	0.057	±0.023	0.091	±0.041	2.009	±0.158	0.144	±0.045
Sa32	0.020	±0.002	0.116	±0.048	0.074	±0.017	0.158	±0.047	0.033	±0.004	0.119	±0.045
Sa33	0.039	±0.007	0.068	±0.007	0.090	±0.029	0.103	±0.029	0.040	±0.016	0.097	±0.029
Sa34	0.023	±0.002	0.043	±0.012	0.033	±0.003	0.061	±0.009	0.008	±0.001	0.087	±0.025
Sa35	0.041	±0.026	0.159	±0.070	0.105	±0.007	0.086	±0.046	0.005	±0.003	0.102	±0.034
Sa36	0.014	±0.010	0.154	±0.030	0.057	±0.004	0.152	±0.014	0.011	±0.004	0.124	±0.035
Sa37	0.013	±0.016	0.603	±0.102	0.092	±0.028	0.086	±0.019	0.024	±0.016	0.069	±0.018
Sa38	0.016	±0.002	0.655	±0.110	0.141	±0.041	0.102	±0.042	0.012	±0.003	0.050	±0.020
Sa39	0.013	±0.002	0.361	±0.021	0.149	±0.046	0.058	±0.019	0.016	±0.005	0.055	±0.005
Sa40	0.009	±0.001	0.106	±0.028	0.056	±0.025	0.084	±0.047	0.026	±0.002	0.082	±0.025
Sa41	0.024	±0.015	0.068	±0.019	0.031	±0.008	0.130	±0.019	0.030	±0.006	0.051	±0.017
Sa42	0.027	±0.013	0.056	±0.031	0.043	±0.008	0.148	±0.028	0.080	±0.017	0.060	±0.010
Sa43	0.004	±0.004	0.216	±0.072	0.178	±0.044	0.046	±0.006	0.020	±0.007	0.111	±0.026
Sa44	0.015	±0.006	0.082	±0.033	0.097	±0.054	0.080	±0.020	0.054	±0.025	0.102	±0.020
Sa45	0.017	±0.002	0.026	±0.002	0.010	±0.001	0.016	±0.005	0.048	±0.005	0.039	±0.008
Sa46	0.017	±0.002	0.026	±0.002	0.010	±0.001	0.016	±0.005	0.048	±0.005	0.039	±0.008

[a]Standard deviation

and *ica*D positive and six were slime producers on CRA.

In this study, we noted that at acidic pH level (pH 3), all the tested strains were biofilm negative (OD_{570} <0.1). Interestingly, at a high alcalinic level (pH 12) all the strains were biofilm negative (OD_{570} <0.1) except for two strains (Sa28 and Sa31), which showed an increase in OD_{570} (0.962 and 2.009 respectively). These strains were *ica*A and *ica*D positive and non slime producers. At this pH, the OD_{570} decreased for the most tested strains compared to that observed at pH 7.

At pH 5, a decrease in biofilm formation in the majority of low-grade biofilm strains was observed. At pH 5, an increase in biofilm formation was observed in six strains (Sa13, Sa22, Sa26, Sa37, Sa38 and Sa39). In addition, at pH 5, 24 strains were classified as low-grade biofilm formation ($0.1 \leq OD_{570} < 1$) and 22 strains were biofilm-negative. On the other hand, at pH 9, the Sa1 as well as 16 of the 28 strains low grade biofilm compared to that observed at pH 7 displayed a decrease in biofilm formation, while two strains (Sa21 and Sa43) showed an increase in biofilm formation and the remaining 27 strains were not inducible.

Effect of hydrogen peroxide on *S. aureus* biofilm formation

In the medium supplemented with 3% hydrogen peroxide, *S. aureus* ATCC 25923 and 17 out of the 46 strains under study were categorized as low-grade positive ($0.1 \leq OD_{570} < 1$) and the 19 strains were non biofilm producers (OD_{570} <0.1) as reported in Table 2. Among the 17 strains low-grade biofilm positive 14 isolates contained *ica*A and *ica*D and nine strains were slime producers on CRA.

In the group of low-grade biofilm-producing strains, 20 out of 28 strains displayed decreased biofilm formation in 3% H_2O_2, while none of the tested strains were inducible with 3% (w/v) H_2O_2 supplementation.

DISCUSSION

Staphylococcus aureus is able to adhere and form biofilm consequently causing severe morbidity and infections (Sheagren, 1989; Waldvogel *et al.,* 1995). Most reports suggested that bacteria in biofilm were more resistant to physical forces such as the shear forces produced by blood flow and the washing action of saliva. Organisms within biofilms can withstand nutrient deprivation, pH changes, oxygen radicals, disinfectants, and antibiotics better than planktonic organisms (Jefferson, 2004).

In this study, we have found that 26 out of 46 strains of *Staphylococcus aureus* (56.5%) were slime producers. In a recent study, Gundogan *et al.,* (2006) found that 58 out of 110 *S. aureus* strains were slime producers. Furthermore, Vasudevan *et al.* (2003) demonstrated that 32 of 35 *S. aureus* isolates were slime positive after 24-48 h. PCR analysis showed that 36 strains tested (78.26%) were positive for both *ica*A and *ica*D genes. Rohde *et al.* (2007) found that all *S. aureus* have the *ica*A gene. On the other hand, Arciola *et al.* (2001) suggested that 60.86% strains of *S. aureus* have the *ica*A and *ica*D. It has been demonstrated that most of the *S. aureus* strains contain the entire *ica* operon (Cramton *et al.,* 1999; Arciola *et al.,* 2001a). In this study, we have found 26 strains positive for icaA/*ica*D were slime producers and 10 were slime non producers. This result was contradictory to those described by Arciola *et al.* (2001b) who suggested that *ica*A and *ica*D were detectable only in slime-producing strains and never in non slime-producing one.

The results of the biofilm formation capacity of *S. aureus* at pH 7 show that only one strain (Sa1) which was *ica*A and *ica*D positive was categorized as highly biofilm-positive ($OD_{570} \geq 1$). These differences may be due to the environmental conditions (Rachid *et al.,* 2000; Cramton *et al.,* 2001, Chaieb *et al.,* 2007). In many biofilm-forming bacteria, the differentiation of planktonic cells into sessile bacteria is associated with environmental stress factors (Costerton *et al.,* 1999, Shapiro, 1998).

At acidic pH level (pH 3), all the tested strains were non biofilm producers (OD_{570} <0.1). Statistical analysis revealed a significant difference between the OD_{570} obtained at pH 7 and pH 3 (P<0.05). These results were in agreement with the study of Memple *et al.* (1998) and Chaieb *et al.* (2007) who found that the biofilm formation of staphylococci cells is inhibited at pH 3. Furthermore, Memple *et al.* (1999) demonstrated that the adherence of *S. aureus* cells is inhibited at acidic pH levels and is enhanced by alka-

line pH values. Our results are in agreement with Hamadi *et al.* (2005) who found that the cells adhere to glass strongly in the pH range 4 to 6 and weakly at highly acidic (pH 2, pH 3) and alkaline pH levels.

On the other hand, at pH 5 and pH 9 and pH12 an increase in biofilm formation was observed in ten strains. As presented in Table 2, at pH 12, two strains (Sa28 and Sa31) were strong biofilm producers, whereas most of the strains were biofilm negative (OD_{570} <0.1). The results are in disagreement with those of White *et al.* (1978) who suggested that the pH of the medium is an important factor in the induction of the *ica*ADBC gene. Moreover, pH has a marked influence on enzyme activity. Each enzyme activity has an optimal pH (White *et al.,* 1978). It has been demonstrated that biofilm formation was induced when grown under pH stress-induced conditions or in the presence of sub-inhibitory tetracycline (Fitzpatrick *et al.,* 2002). Statistical analysis revealed a significant difference between the OD_{570} obtained, at pH 7-pH 9 as well as at pH 7-pH 12 (P<0.05). However the difference observed between the OD_{570} at pH 7 and that pH 5 was not significant (P=0.300).

In this study, no correlation between slime production in CRA and biofilm formation was noted. We also found that some strains positive for *ica*A and *ica*D were biofilm positive by the microtiter plate assay and were non producers on the CRA plates.

For biofilm formation, synthesis of an intercellular polysaccharide adhesin (PIA) is necessary to mediate cell-to-cell adhesion. This PIA is synthesized by the gene products of the *ica*ADBC locus (Cramton *et al.,* 1999, Mckenney *et al.,* 1999). Cramton *et al.* (1999) suggested that biofilm-negative phenotype in *S. aureus* resulted from the deletion of the *ica* operon. The initial adhesion of bacterial cells to the polymer surface was influenced by the environmental conditions (Chaieb *et al.,* 2007). However, the microbial initial adhesion phenomenon cannot be achieved without considering the effect of the substrate, various properties of the cell surface and characteristics of the aqueous medium, such as ionic strength, temperature and pH (Hamadi *et al.,* 2004).

In this study the biofilm formation observed at alkaline pH (pH 12) and at acidic pH (pH 5) may be due to weak electrostatic repulsion between cells to cell and the substrate. It has been reported that the reduction of spore-spore electrostatic repulsion favours formation of aggregates (Gerin *et al.,* 1995). Secondly, teichoic acid is responsible for maintaining the net negative charge of Gram-positive bacteria that prevents aggregation with other bacteria (Heptinstall *et al.,* 1970). However, this phenomenon could be explained by the fact that the electrostatic properties of polystyrene surfaces may be influenced by pH value (Meinders *et al.,* 1994).

Bacteria in biofilms resist antibiotics, biocides, and disinfectants in comparison to free-floating cells (Xu *et al.,* 2000). We investigated the efficacy of 3% hydrogen peroxide on biofilm formation of *S. aureus*.

In this study at 3% H_2O_2, in the group containing the low-grade biofilm-producing strains, 20 out of 28 displayed decreased biofilm formation and none of the tested strains were inducible with 3% H_20_2 supplementation. Statistical analysis revealed a significant difference between the OD_{570} obtained at pH 7-(3% H_2O_2) (P<0.05). Pitts *et al.* (2003) showed that hydrogen peroxide removed *Staphylococcus epidermidis* biofilm more effectively than *Pseudomonas aeruginosa* biofilm. In addition, *S. aureus* ATCC 25923 and 17 of the 46 strains were classified as low-grade biofilm-positive (0.1≤OD570 <1). Eckhard *et al.,* (1999) found that local treatment with 3% H_2O_2 significantly reduced bacterial growth by more than 99% on polymer biomaterials. This finding might influence clinical strategies of preventing foreign body infection.

However, we noted that *S. aureus* ATCC 25923 and 17 of the 46 strains were biofilm producers at 3% H_2O_2. Poor antimicrobial penetration in biofilm has been described for hydrogen peroxide (Stewart *et al.,* 2000) which is degraded by catalases, and beta-lactam antibiotics (Anderl *et al.,* 2000), which are cleaved by beta-lactamases.

In conclusion, biofilm formation was largely affected by the pH levels and H_2O_2 suspending medium supplementation. Our study also confirms the biofilm variations in clinical *S. aureus* strains. However, biofilm formation was reduced when strains were grown under 3% H_20_2 and at very low pH. Furthermore, the induction of biofilm formation for some studied strains was observed at pH (5, 9 and 12). In addition, environmental conditions may also confer a positive

selective pressure on biofilm-positive *S. aureus*, promoting the occurrence of medical device-related infections.

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