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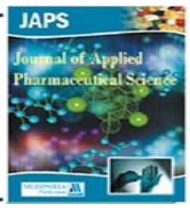

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In-vitro adhesion of *Staphylococcus* spp. to certain orthopedic biomaterials and expression of adhesion genes

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ABSTRACT

The present study evaluated biofilm forming capacity, the adherence of Staphylococci spp. to different orthopedic biomaterials and the presence of both *icaA* and *icaD* genes among staphylococci strains isolated from patients suffering from orthopedic implant infections. We studied 53 *Staphylococcal* strains from infections related to orthopedic implants, as regards their ability to form biofilm by using microtitre plate method (MTP), in vitro evaluation of the ability of the biofilm forming strains to adhere to certain biomaterials that used in orthopedic surgery and detection of *ica A* and *ica D* among the isolates. 90.9% of *S. aureus* strains were biofilm positive while, 95% of Coagulase negative staph. were biofilm forming, PMMA demonstrated a significantly highest adherence ($P<0.05$) followed by stainless steel while, the lowest adherence exhibited by titanium and Biofilm producing strains were positive for *icaA* and *icaD* genes while, biofilm negative strains were negative for both genes. *Staphylococcus* spp. are the major pathogens in orthopedic implants infections. Titanium biomaterials are less susceptible for adherence by bacteria . Biofilms are considered the key factor in the development of implant-related infections.

Keywords: Adhesion, orthopedic biomaterials, *Staphylococci* and slime.

INTRODUCTION

More than half of prosthesis-associated infections are caused by *S. epidermidis* and *S. aureus* (Zimmerli and Ochsner, 2003) with biofilm formation representing a major step in their pathogenesis. Biofilm offers protective barrier to organisms, resulting in resistance to antimicrobial agents (Darouiche *et al*., 1994) and host immune responses (Brady *et al*., 2006; Bjarnsholt *et al*., 2008). Recently, the genetic control of the slime production has been determined (Mckenney *et al*., 1999). Synthesis of the capsular polysaccharide is mediated by the *ica* operon. On activation of this operon, a polysaccharide intercellular adhesin (PIA) is synthesized. This supports cell-to-cell bacterial contacts by means of a multilayered biofilm. The PIA is composed of linear ß-1,6-linked glucosaminylglycans.

It is synthesized in vitro from UDPN- acetylglucosamine by the enzyme N-acetylglucosaminyltransferase, which is encoded by the intercellular adhesion (*ica*) locus and, in particular, by the *icaA* gene. Sole expression of *icaA* induces only low enzymatic activity, but co-expression of *icaA* with *icaD* significantly increases the activity and is related to the phenotypic expression of the capsular polysaccharide (Gerke *et al.*, 1998).

In routine orthopedic surgery, several different foreign materials are regularly implanted, e.g. bone cement, polyethylene compounds, and different metal alloys. Biomaterials have different affinities for bacteria (Oga *et al.*, 1993). In general, an increase in surface roughness enhances bacterial colonization and early biofilm formation (Arnold and Bailey, 2000).

In this study we investigated incidence of biofilm formation among *Staphylococcus* spp. isolated from patients with orthopedic implants, in vitro evaluation of biofilm formation on polymethylmethacrylate (PMMA) bone cement and certain metallic biomaterials and the occurrence of *icaA* and *icaD* genes for slime production in a collection of *S. epidermidis* clinical isolates by a simple, rapid and reliable polymerase chain reaction (PCR).

MATERIALS AND METHODS

Bacterial Strains

53 clinical isolates of *Staphylococcus* spp. were included in the study. The strains were isolated from orthopedic implants (prostheses, plates, pins and screws, intramedullary nails and fragments of wires used in Illiazarov devices from 83 consecutive patients suffering from orthopedic implants infections in the department of orthopedics at Minia university hospital. The isolates were identified by conventional biochemical tests.

ORTHOPEDIC BIOMATERIALS

Commercially available Kirschner-wires (K-wires) and screws of stainless steel and titanium: Suzhou ideal Medical Instrument Co. Ltd., France and Cemex® PMMA (poly methyl methacrylate) (bone cement): Tecres medical high technology, Verona, Italy.

PHENOTYPIC AND QUANTITATIVE CHARACTERIZATION OF BIOFILM FORMATION USING MICROTITRE PLATE (MTP) (Christensen *et al.*, 1985)

Staphylococcus isolates were screened for their ability to form biofilm. Organisms isolated from fresh agar plates were inoculated in 10 ml of trypticase soy broth with 1% glucose. Broths were incubated at 37°C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates were filled with 200 µl of the diluted cultures. Negative control wells contained inoculated sterile broth. The plates were incubated at 37°C for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with0.2 ml of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. 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 biofilm was obtained by using micro ELISA autoreader at wavelength 570 nm. The experiment was performed in triplicate and repeated three times. To compensate for background absorbance, OD readings of wells with ethanol were used as blank and subtracted from all tests' values. Biofilm production is considered high (> 0.240), moderate (0.120 - 0.240) or weak/none (< 0.120).

IN VITRO DEVELOPMENT OF BACTERIAL BIOFILMS ON ORTHOPEDIC BIOMATERIALS (Bahna *et al.*, 2007)

Comparison of biofilm formation on the following three different biomaterials was done.

- Titanium *Kirschner* wires (1 cm length).
- Stainless steel *Kirschner* wires (1 cm length).
- PMMA (poly methyl methacrylate) (bone cement): PMMA bone cement was prepared in accordance with the manufacturer's instructions by mixing the powdered methyl methacrylate with the liquid monomer in a bowl using a spatula. The cement mixture was immediately placed between two glass plates covered with nonadhesive backing paper, which were pressed together to form a sheet of cement approximately 1mm thick. Following hardening of the cement, 1 cm^2 sections were cut with a sterile scalpel blade and stored under dark, sterile conditions at room temperature (Ramage *et al.*, 2003).

These biomaterials were placed in 1ml of donor calf serum and incubated overnight at 37 ◦C. biomaterials were then immersed in 1ml of Mueller–Hinton broth inoculated with 5.5×10^5 CFU/ml of a clinical biofilm-forming bacterial isolates and incubated overnight at 37 ◦C. The broth was then replaced with 1ml of 0.9% sterile saline and washed with shaking for 30 min. to discard any planktonic bacteria. Without disurbing the biofilm, the biomaterials were then transferred to 5mL of 0.9% saline, sonicated to dislodge the bacterial biofilm for 15 min and vortexed for 30 s. An aliquot of 100 µl was spread onto trypticase soy agar with 5% sheep blood, incubated for 24 h at 37 ◦C and then counted. A value of 100 CFU was used for any plate that had at least 100 colonies. Final colony counts were then calculated accounting for the dilution factor.

SCANNING ELECTRON MICROSCOPE (SEM) (Hudetz *et al.*, 2008)

Orthopedic biomaterials (PMMA sections, stainless steel and titanium wires) were fixed in 2.5 % (v/v) glutaraldehyde in Dulbecco PBS (PH 7.2) for 1.5 h., rinsed with PBS, and then dehydrated through an ethanol series. Samples were critical point

dried and gold-palladium coated. SEM examinations were made on JSM-840 SEM.

PCR DETECTION OF *ICA A* AND *ICA D* GENES IN SLIME PRODUCING *STAPHYLOCOCCI* STRAINS

Bacterial DNA extraction (Seif El-Din *et al.*, 2011)

Bacterial DNA was extracted from *staphylococcal* pure colonies grown on blood agar and suspended in nutrient broth using QIAamp Mini DNA extraction kit according to the manufacturer's instructions.

PCR method for amplification of *icaA* and *icaD* sequences (Arciola *et al.*, 2001)

For the detection of *icaA* gene 5-TCTCTTGCAGGAGCAATCAA-3 was used as the forward primer (corresponding to nucleotides 4796–4815) and 5-TCAGGCACTAACATCCAGCA-3 was used as the reverse primer (corresponding to nucleotides 4964-4983). For *icaD*, 5-ATGGTCAAGCCCAGACAGAG-3 was used as the forward primer (corresponding to nucleotides 5422–5441), and 5-CGTGTTTTCAACATTTAATGCAA-3 was used as the reverse primer (corresponding to nucleotides 5616–5597). Reaction mixtures (50 µl) contained 25 µl PCR master mixtures, 1 µl of each primer (0.1-0.5 µM final concentration), 18 µl RNA ase free water & 5 µl of template DNA. Amplifications were performed with the following thermal cycling profile an initial denaturation at 94°C for 2 min., followed by 30 cycles of amplification (denaturation at 94°C for 1 min., primer annealing at 60°C for 1 min., and extension at 72°C for 2 min.) and a final extension for 4 minutes. After amplification, 10 µl of the PCR mixture was analyzed by agarose gel electrophoresis. Amplicons for *icaA* and *icaD* produced fragments of 188 and 198 bp, respectively. The amplified product sizes were estimated by comparison with 100 bp DNA ladder.

STATISTICAL ANALYSIS

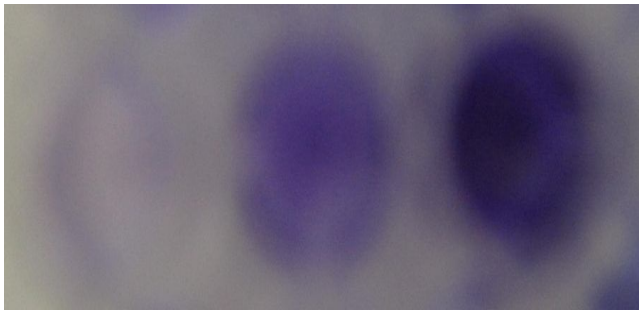
One-Way ANOVA to evaluate any significant difference between values viable bacterial counts among different biomaterials but unpaired t-test was used to evaluate any significant differences of viable bacterial counts among stainless steel and titanium. Differences were done using graphpad prism 5 software. P values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Phenotypic and quantitative characterization of biofilm formation using microtitre plate method (MTP)

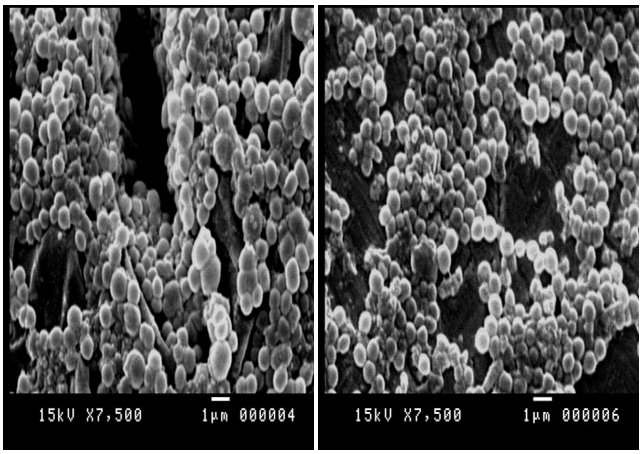
Biofilm production assessed by MTP revealed that 30 (90.9%) strains of *S. aureus* were biofilm positive and 19 strains (95%) of Coagulase negative staph. were biofilm forming. Quantitative biofilm production showed that 22 (76.7%) strains were strong biofilm producers with readings > 0.240, 8 (24.2%) strain was moderate biofilm producer, with readings 0.120 - 0.240 and 3 (9.1%) strains were non-biofilm producers with readings < 0.120.

In CONS, 13 (65%) strains, were strong biofilm producers, 6 (30%) was moderate biofilm producers and One (5%) strains were non-biofilm producers .



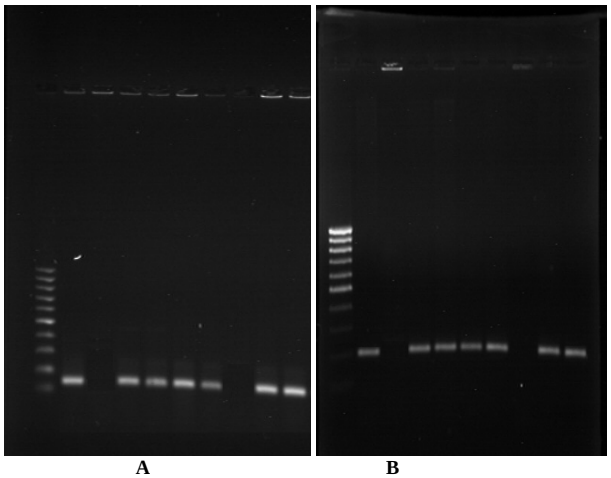
A B C

Fig. 1: Quantitative detection of biofilm production by MTP- high (C), moderate (B) and non slime producers (A) differentiated by crystal violet staining in 96 well microtiter plates.



A B

Fig. 2: SEM of in vitro adherence of *Staphylococcus* spp. to PMMA sections (biofilm was shown to be composed of many multilayered bacterial colonies, forming different sized colony Masses) (A) and metallic biomaterials (less biofilm layer was observed) (B) (× 7500).



A B

Fig. 3: PCR detection of *icaA* (A) and *icaD* (B) genes. Lane 1, molecular size marker (100 bp Ladder); lanes 2, 4, 5 , 6, 7, 9 and 10, *icaA* at 188 bp and *icaD* at 198 bp; lane 8,3 negative biofilm (DNA template absent).

Table. 1: *Staphylococci* spp. adherence to different orthopedic biomaterials:

Bacteria	Strain no.	PMMA (CFU/ml)	Metallic biomaterials (CFU/ml)			P value
			Stainless steel	Titanium	P value	
S. *aureus*	1	2×10^5	48×10^3	32×10^2	P<0.05	P<0.05
	2	5×10^5	2×10^5	14×10^3		
	3	17×10^5	5×10^5	23×10^3		
	4	83×10^4	32×10^4	9×10^3		
	5	17×10^5	2×10^5	31×10^3		
Coagulase negative *staph.*	6	16×10^5	5×10^4	8×10^3	P<0.05	P<0.05
	7	74×10^4	32×10^4	29×10^2		
	8	69×10^4	22×10^4	9×10^3		
	9	87×10^4	51×10^3	38×10^2		
	10	94×10^4	58×10^4	5×10^3		

***In-vitro* development of bacterial biofilms on certain orthopedic biomaterials**

Significant differences (P<0.05) were observed in the adherence of bacterial isolates to each of the biomaterials. PMMA demonstrated a significantly highest adherence (P<0.05) followed by stainless steel while, the lowest adherence exhibited by titanium.

PCR detection of ica A and ica D genes in slime producing Staphylococci strains

The presence of adhesion genes (*ica A* and *ica D*) were investigated in biofilm forming and non forming *Staphylococcal* strains. These biofilm producing strains isolated from orthopedic implants were found to be positive for both genes, giving a 188-bp band for *icaA*, and a 198-bp band for *icaD* genes. It was also found that these strains which were positive for *icaA* were also positive for *icaD* but absence of both genes in non biofilm forming strains.

DISCUSSION

In the present study we have assayed isolated staphylococcal strains implant isolates for qualitative biofilm forming ability by microtitre plate method. There were 90.9 % strains of *S. aureus* and 95% of CONS biofilm forming. Similar results were reported by Bartoszewicz *et al.* (2007) who found that most of 16 strains (*E. coli, S. aureus, S. epidermidis and enterobacter*) isolated from metal orthopedic components were able to form a biofilm. However Arciola *et al.* (2005) reported that among clinical 342 isolates of *S. epidermidis* from orthopedic infections, 126 (36.8%) were identified as exopolysaccharide-forming strains strains, while 216 (63.2%) were found to be CRA-negative using Congo Red Agar (CRA) test. This study described the formation of *Staphylococcal* isolates biofilm on different orthopedic biomaterials (bone cement, stainless steel and titanium). Significant differences (P<0.05) were observed in the adherence of bacterial isolates to each of the biomaterials. PMMA demonstrated a significantly highest adherence (P<0.05) followed by stainless steel while, the lowest adherence exhibited by titanium. These differences in the ability of adhesions may be due to possessing different surface finish and therefore a different surface roughness. Similar results were obtained by Arens *et al.* (1996) who demonstrated lower rates of infection for titanium dynamic compression plate (DCP) compared to stainless steel DCP in the presence of a local bacterial challenge In an animal experiment with statistical significance.

Sheehan *et al.* (2004) studied pre- and direct inoculation with *Staphylococcus aureus* and *epidermidis* on titanium and stainless steel metallic implants in rabbits, it was found that *Staphylococcus epidermidis* showed lower adhesion ability to metals, and biofilms adhered in greater numbers to stainless steel over titanium. These results are close to the present findings.

Petty *et al.* (1985) Confirmed the present results in an animal model by measuring the (ID_{50} dose), the amount of microorganisms necessary to cause an implant associated infection in 50% of dogs. Stainless steel, PMMA, cobalt chromium alloy and other biomaterials were inserted in vivo in animal femora and infected with *Staphylococcus aureus*, *epidermidis* and *enterococcus* sp. PMMA reduced the ID_{50} compared to other biomaterials.

The presence of both *ica* A and *ica* D genes in biofilm forming Staphylococci spp. is supported by the results of a study done by Arciola *et al.* (2003). These findings are consistent with those of other studies, which showed a high incidence of slime producing Staphylococci in isolates from infections of different original (El-Mahallawy *et al*., 2009; Seif El-Din *et al.*, 2011).

CONCLUSION

PMMA demonstrated a significantly highest adherence followed by stainless steel while, the lowest adherence exhibited by titanium. These findings should be put into consideration regarding susceptibility of the implants to microbial infection and adhesion. Our findings indicate an important role of ica genes as a virulence marker for *Staphylococcal isolates*. its association with the biofilm forming strains strongly suggest that expression of *icaA* and *icaD* genes plays a role in the pathogenetic mechanisms of infection associated with orthopedic implants

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