Full Length Research paper

Evaluation of biofilm production by *Staphylococcus epidermidis* isolates from nosocomial infections and skin of healthy volunteers

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***Staphylococcus epidermidis* is a frequent cause of nosocomial infections. The major virulence factor is thought to be biofilm formation by the organism mediated by gene products of the *icaADBC* operon. In this research, biofilm phenotype and *icaADBC* gene carriage were studied in 50 *S. epidermidis* isolates from symptomatic patients (group A) and 50 skin isolates from healthy individuals (group B). Biofilm phenotype was shown by colony morphology on Congo red agar and the microtiter plate method was used for quantitative measurement of biofilm formation. Polymerase chain reaction was employed to detect the presence of *icaADBC* operon. The results showed no significant difference between the two groups of isolates for the potential to form biofilms by the two phenotypic assays or the amounts of biofilm produced by the two groups of isolates. On the other hand, *ica* gene carriage was more discriminatory and was observed in 30% of group A isolates compared to 8% of the skin isolates. We conclude that *S. epidermidis* isolates from patients with symptomatic infections are not necessarily more virulent from the skin contaminants and the capacity to form biofilms *in vivo* is influenced by environmental stimuli independent of the *icaADBC* gene products.**

Key words: Biofilm, *Staphylococcus epidermidis*, *icaADBC*, patients isolates, skin isolates.

INTRODUCTION

Coagulase negative staphylococci (CoNS) are part of the normal skin and mucosal microflora. However, in recent years, *S. epidermidis* has emerged as a common cause of nosocomial infections in immunocompromised patients (Rupp and Archer, 1994; Kloos and Bannerman, 1994). Septicaemia due to *S. epidermidis* is often associated with the use of catheters and other indwelling medical devices (Kloos and Bannerman, 1994; Pfaller and Herwaldt, 1988; Peters, 1986). The major pathogenic factor of *S. epidermidis* is thought to be biofilm formation mediated by a Polysaccharide Intercellular Adhesin (PIA). In fact, the important role of biofilm formation in the pathogenesis of *S. epidermidis* bio-material related infec-tions has been documented in animal models (Rupp et al., 1999 a and b). PIA is a β- 1, 6- linked N- acetylglucos-amine polymer responsible for cell-cell attachment (Gerke et al., 1998; Heilmann et al., 1998). PIA produc-tion in S. *epidermidis* is encoded by the *ica* operon consisting of *icaA*, *icaD* , *icaB* and *icaC* genes and is regulated by the product of *icaR* . *IcaA* and *D* encode N-acetylglucoseaminyl transferase which synthesizes the PIA polymer, *icaC* is responsible for formation of long polymer chains and *icaB* deacetylates the poly-N-acetyl-glucoseamine molecule (Gerke et al., 1998; Freeman et al., 1989) . The *in vitro* amounts of biofilm produced by individual strains of S. *epidermidis* are highly variable and greatly influenced by glucose and other environmental and growth conditions (Mack et al., 1992; Cramton et al., 2001; Rachid et al., 2000; Cerca et al., 2004; Eftekhar and Speert, 2009).

This study was carried out to compare and evaluate the significance of biofilm production and presence of the *ica* operon in S. *epidermidis* isolates recovered from patients

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admitted to hospitals with various CoNS infections as well as those from the skin of healthy subjects.

MATERIALS AND METHODS

Bacterial strains

Fifty isolates of *Staphylococcus epidermidis* were collected from patients admitted to three Tehran hospitals (Taleghani, Emam Hosein and Booali) (group A). The specimens were mostly from blood (40%) followed by urine (14%), surgical wounds (14%), intravascular catheters (8%), exudates (8%) and other unknown sources (16%). Fifty isolates were also collected from the nasal passage or nearby skin regions of healthy individual volunteers using a moistened swab (group B). Identification of *S. epidermidis* was carried out using the standard biochemical tests including catalase, DNase and coagulase production, growth and fermentation of mannitol on mannitol salt agar and susceptibility to bacitracin and novobiocin (Bannerman and Peacock, 2007). Bacteria were maintained in Lauria Bertani broth (LB) containing 8% DMSO at –80 °C. Biofilm positive *S. epidermidis* strain RP62A and its isogenic biofilm negative mutant RP62NA were kindly provided by Dr. Gerald Pier (Harvard Medical School, Boston, MA).

Phenotype analysis of biofilm production on CRA

Biofilm forming colony morphology was detected for *S. epidermidis* isolates on CRA plates containing 21 g Mueller–Hinton broth, 15 g granulated agar; 36 g sucrose and 0.8 g Congo red per liter of distilled water (Handke et al., 2004). Bacteria were cultured in 10 ml tryptic soy broth at 35 °C for 24 h without shaking, and were then plated onto CRA plates. Incubation was carried out at 35 °C for 24 h and an additional 24 h at room temperature before recording the colony morphology. Crusty black colonies with dry filamentous appearance were recorded as biofilm producers, smooth pink colonies as non-producers and intermediate colony morphology (pink with dark centers resembling bull's eyes) as potential biofilm producers (Aricola et al., 2005).

Quantitative determination of biofilm production

Biofilm production was carried out as described previously (Eftekhar and Speert, 2009). Briefly, overnight grown bacteria in Trypticase Soy Broth (TSB) were diluted (1:100) and 200 μl portions were inoculated into 96-well flat bottom polystyrene microtiter plates (Cellstar, greiner bio-one). Incubation was carried out at 35 °C for 22 - 24 h before removal of the cultures. The wells were washed 3 times with phosphate buffered saline (PBS, pH, 7.2), air dried and stained with 0.1% safranin. The optical density of the wells was measured at 490 nm using micro Elisa auto reader (Stat Fax 2100, Awareness Technol. Inc). An optical density of 0.12 was chosen to distinguish biofilm producers from those that did not form biofilm. Biofilm-positive and negative strains of *S. epidermidis* were included in each plate as was a negative control of medium without bacteria. The tests were carried out in quadruplicate and all strains were tested on at least two different days.

Detection of *icaADBC* genes

Genomic DNA was extracted by boiling as previously described (Pérez-Roth, 2001). Briefly, several colonies from an overnight grown culture on nutrient agar were resuspended in 250 μl ddH$_2$O and placed in a boiling water bath for 20 min before centrifugation at 12000 x g for 5 min. Detection of the *ica ADBC* gene cluster was carried out by amplification of a DNA region partially spanning the *icaA*, *icaD* and *icaB* genes to yield a 546 bp amplification product (Eftekhar and Speert, 2009). The primers used were: *icaADB*-F 1893-TTATCAATGCCGCAGTTGTC-1913 and *icaADB*-R 2388-GTTTAACGCGAGTGCGCTAT-2408. Reaction mixtures (25 μl) contained 10 μl genomic DNA, 40 pM of each oligonucleotide primer, 0.2 mM dNTP mix, 1.25 u Taq polymerase (Cinnagen, Iran), 10 mM Tris-HCl (pH 8.3) and 1.5 mM MgCl$_2$. Amplifications were performed using a Bioer Little Genius thermal cycler with the following thermal cycling profile: an initial denaturation at 94°C for 5 min followed by 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 55 C for 30 s and extension at 72°C for 1 min) and a final extension period of seven min at 72°C.

RESULTS

The results of colony morphology on CRA plates showed that among group A isolates, 12(24%) formed black colonies, 18(36%) were non-producers (pink colonies) and 20(40%) had the intermediate colony morphology for biofilm formation. There was no relation between CRA colony morphology and the infection site where the organism was isolated. Among group B isolates, 12(24%) formed black colonies, 11(22%) produced pink colonies and 22(44%) had the intermediate appearance. Taking into account the intermediate colony morphology, 64% of group A and 68% of group B isolates had the potential to form biofilm, suggesting no difference between the two categories.

Using the microtiter method (Mtp), 26(52%) of group A and 28(56%) of group B isolates were biofilm producers. There was no significant difference between the capacity to produce biofilm or the quantities of biofilm formed by the organisms from the two groups (Figure 1) . Among the 42 isolates in group A which came from the known infection sites, 50% of the blood isolates (10/20), 75% of the isolates from catheters (3/4), 50% of the isolates from exudates (2/4) and 14.5% of the wound (1/7) or urinary isolates were biofilm producers. However, given the small number of samples from different infections, it is not possible to conclude whether there was a relationship between the source of infection and biofilm formation.

The *ica* gene carriage was observed in 15(30%) of group A isolates among which, 7(14%) were biofilm producers by both phenotypic methods, 5(10%) did not have biofilm phenotype in either of the test assays, 2(4%) had the Mtp phenotype and 1(2%) was CRA positive. Similar to the MTp results, *ica* gene carriage by the isolates could not be related to the infection site. Among group B isolates, 4(8 %) carried the *ica* operon and all had the biofilm producing phenotype using both assays. There was a better agreement between *ica* gene carriage and colony morphology on CRA plates compared to the microtiter method (Table 1).

DISCUSSION

In recent years, *S. epidermidis*, has been recognized as a major nosocomial pathogen, mainly due to its ability to
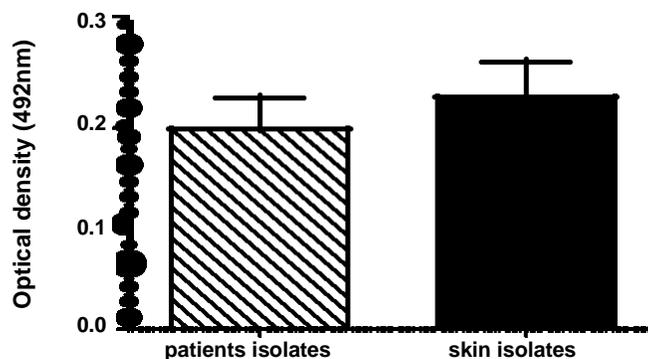
Figure 1. Biofilm formation by clinical isolates of *S. epidermidis*; 50 from symptomatic patients and 50 normal skin isolates using the microtiter plate assay.

Table 1. Correlation of biofilm formation and *icaADB* gene carriage by isolates of S. *epidermidis* recovered from symptomatic patients and from healthy volunteers.

	% Correlation between		
S. epidermidis	Mtb[a] /*icaADB*	CRA[b] /*icaADB*	Mtb[a]/CRA[b]
Symptomatic patients (n = 50)	66	86	58
Skin of healthy subjects (n = 50)	52	82	62

[a]Mtb, microtiter plate, % organisms in which biofilm phenotype correlated with *ica* gene carriage (biofilm producers/ *ica* +or biofilm negative isolates / *ica* -).
[b]Congo red agar, % organisms in which colony morphology correlated with *ica* gene carriage (pink/*icaADB*-, black/*icaADB* +).

adhere and produce biofilms on catheters and other foreign bodies. Detection of the *ica* locus in S. *epidermidis* has been suggested to be a good predictor of biofilm formation for distinguishing blood and catheter-related infecting organisms from contaminating bacteria (Aricola et al., 2005; Ziebuhr et al., 1997; Galdbart et al., 2000). On the other hand, some investigators have found no association between the presence of the *ica* operon and biofilm formation by clinical isolates of S. *epidermidis* (Ninin et al., 2006; de Silva et al., 2002).

In addition, expression of the *ica* m-RNA has been shown to occur in biofilm negative *S. epidermidis* or bio-film producing strains under experimental conditions which did not promote biofilm formation, suggesting that biofilm accumulation is controlled by regulatory mecha-nisms other than the *ica* operon (Dobinsky et al., 2003). We found no difference in the capacity to form biofilms, by *S. epidermidis* isolates from symptomatic patients compared to skin isolates from healthy volunteers using two phenotypic methods. On the other hand, 8% of the skin isolates carried the *ica* operon compared to 30% of the patient's isolates suggesting that the *ica* operon was the only discriminatory factor among the two groups in our experiments.

Comparison of biofilm formation by the two phenotypic methods and *ica* gene carriage showed that there was a better agreement between presence of the *ica* operon and colony morphology (86% for group A and 82% for group B isolates) compared to the results obtained for *ica* gene carriage in relation to the Mtp method (74% for group A compared to 62% group B isolates). Of the 50 group A isolates, 42 were from known infection sites, among which, good agreement was observed between *ica* gene carriage and colony morphology on CRA plates (*ica* positive/ black colonies, or *ica* negative/ pink colonies) in 90% of blood (18/20) and 100% of the rest of group A isolates. On the other hand, 60% of blood isolates (12/20), 71.1% of urinary isolates (5/7), 75% of the isolates from exudates (3/4) and catheters (3/4) and 100% of the wound isolates (7/7) were *ica* positive/ Mtp positive, or *ica* negative/ Mtp negative. Our results agree with the finding by Aricola in that there was a better agreement between CRA plate method and *ica* gene carriage compared to the Mtp method (Aricola et al., 2005).

Overall, *ica* gene carriage was more discriminatory among the two groups of isolates compared to the phenotypic methods employed. We conclude that *S. epidermidis* isolates from patients with symptomatic infections are not necessarily more virulent (pathogenic) than the skin contaminants and the capacity to form biofilms *in vivo* is influenced by environmental stimuli, ex-

pression levels of *icaADBC* or other regulatory factors independent of PIA synthesis.

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