Indian J Med Res 138, August 2013, pp 262-266

Glucose & sodium chloride induced biofilm production & *ica* operon in clinical isolates of staphylococci

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Received March 9, 2011

***Background & objectives*: All colonizing and invasive staphylococcal isolates may not produce biofilm but may turn biofilm producers in certain situations due to change in environmental factors. This study was done to test the hypothesis that non biofilm producing clinical staphylococci isolates turn biofilm producers in presence of sodium chloride (isotonic) and high concentration of glucose, irrespective of presence or absence of *ica* operon.**

***Methods*: Clinical isolates of 100 invasive, 50 colonizing and 50 commensal staphylococci were tested for biofilm production by microtiter plate method in different culture media (trypticase soy broth alone or supplemented with 0.9% NaCl/ 5 or 10% glucose). All isolates were tested for the presence of *ica ADBC* genes by PCR.**

***Results*: Biofilm production significantly increased in the presence of glucose and saline, most, when both glucose and saline were used together. All the *ica* positive staphylococcal isolates and some *ica* negative isolates turned biofilm producer in at least one of the tested culture conditions. Those remained biofilm negative in different culture conditions were all *ica* negative.**

***Interpretation & conclusions*: The present results showed that the use of glucose or NaCl or combination of both enhanced biofilm producing capacity of staphylococcal isolates irrespective of presence or absence of *ica* operon.**

Key words Biofilm production - glucose - *ica* operon - NaCl - staphylococci

Staphylococci are common cause of hospital acquired infections and biofilm is one of its important virulence factors[1,2]. Its production is dependent on polysaccharide intracellular adhesin (PIA) synthesis. Regulation of PIA synthesis and biofilm production by microbes is a complicated process and is influenced by many factors, *e.g.* constitutional microbial factors, environmental factors and in clinical situations host proteins, *etc*. The enzymes involved in PIA synthesis are encoded by the *ica* operon comprising *icaA, icaD, icaB,* and *icaC* genes. Expression of the *ica* operon and formation of biofilm are highly variable[3] and *ica* negative biofilm positive strains of staphylococci are also known to have alternative regulatory mechanism of biofilm formation[4]. All colonizing and invasive staphylococcal isolates may not produce biofilm in some situations but may turn biofilm producers in other situations due to change in environmental factors[5-7]. This study

was planned to test the hypothesis that non biofilm producer clinical isolates of staphylococci turn biofilm producers in presence of sodium chloride (isotonic) and high concentration of glucose, irrespective of presence or absence of *ica* operon.

Material & Methods

Isolation and identification of staphylococci was done as reported in our previous study[8]. The isolates were grouped in three categories: *(i)* Invasive isolates (100): Isolates obtained from two consecutive blood cultures of same patient. *(ii)* Colonizing isolates *(*50): Isolates from peripheral intravenous device (IVD) of the patients whose blood culture was negative for *Staphylococcus*. *(iii)* Commensal isolates (50): Staphylococcal isolates from skin and or nasal swab of patients, whose blood culture and peripheral IVD culture were negative for *Staphylococcus*.

Detection of biofilm: Isolates of *S. aureus* and coagulase negative staphylococci (CNS) were studied for biofilm producing capacity by microtiter plate method[9] at 37° C, aerobically in the following culture media; A= Trypticase soy broth (TSB) alone, B= TSB+5 per cent glucose, C= TSB+10 per cent glucose, D= TSB+0.9 per cent NaCl, E= TSB+5 per cent glucose+0.9 per cent NaCl, F= TSB+10 per cent glucose+0.9 per cent NaCl.

Detection of ica ABDC genes: DNA from four to five colonies of each isolate was extracted[10] and amplification of *ica ADBC* gene was done by using the protocol of Zeibhur *et al*[11].

Statistical analysis: Statistical analysis was done using SPSS (Statistical Package for Social Scientists) software of 15.0 version, USA. Chi square test was used for comparison of proportion.

Results & Discussion

Of the 200 isolates, 139 were *S. aureus* and 61 were CNS. Of the 100 invasive isolates, 84 were *S. aureus* and 16 were CNS (3 *S. epidermidis*, 13 *S. haemolyticus*); of the 50 colonizing isolates, 30 were *S. aureus* and 20 were CNS (17 *S. epidermidis*, 3 *S. xylosus*), and of the 50 commensal isolates, 25 were *S. aureus* and 25 were CNS (23 *S. epidermidis*, 2 *S. saprophyticus*).

Of the 84 invasive *S. aureus* isolates, 67 (79%) showed biofilm producing potential, followed by colonizing 73 per cent (22/30) and commensals 28 per cent (7/25) isolates ($P<0.001$ among groups). All three (100%) invasive *S. epidermidis* isolates were biofilm producers followed by colonizing [70.5% (12/17)] and commensal isolates [39.1% (9/23)]. Though all *S. haemolyticus* isolates were invasive, but only 30.7 per cent (4/13) were able to produce biofilm. *S. saprophyticus* and *S. xylosus* both were non biofilm producing.

***Staphylococcus aureus*:**

Invasive isolates - The mean absorbance value in different culture conditions increased from 0.386±.05 (control) to 1.67±.20 (highest in presence of both glucose and NaCl). Of the 67 biofilm positive invasive isolates, 63 were *ica* positive (53 *ica ADBC* positive, 10 *ica AD* positive) (Table I). Of the 17 biofilm negative isolates, 15 turned biofilm positive (10 *ica ADBC* positive, 3 *ica AD* positive, and 2 *ica* negative) in presence of either glucose or sodium chloride or both. Two isolates remained biofilm negative (both *ica* negative) (Table II).

Colonizing isolates - The mean absorbance value in different culture conditions increased from 0.377±.02 (control) to 1.23±.10 (highest in presence of both glucose and NaCl). All 22 biofilm positive isolates were

Table I. *ica* operon in biofilm positive and negative staphylococcal isolates

Groups	*S. aureus* (n=139)						CNS (n=61)					
	Biofilm +ve			Biofilm –ve			Biofilm +ve			Biofilm -ve		
	N	*ica* +ve	*ica* –ve	N	*ica* +ve	*ica* –ve	N	*ica* +ve	*ica* -ve	N	*ica* +ve	*ica* –ve
Invasive	67	63	4	17	13	4	7	7	0	9	7	2
Colonizing	22	22	0	8	3	5	12	10	2	8	5	3
Commensal	7	4	3	18	2	16	9	6	3	16	2	14
Total	96	89	7	43	18	25	28	23	5	33	14	19

CNS, coagulase negative staphylococci; N, number tested; +ve, positive; -ve, negative

ica positive (15 *ica ADBC* positive, 7 *ica AD* positive) (Table I). Of the eight biofilm negative isolates, five turned biofilm positive (1 *ica ADBC* positive, 2 *ica AD* positive and 2 *ica* negative) in presence of glucose/ NaCl/ both, however, 3 remained biofilm negative (all *ica* negative) (Table II).

Commensal isolates - The mean absorbance value in different culture conditions increased from 0.276±.02 (control) to 1.23±.10 (highest in presence of both glucose and NaCl). Of the seven biofilm positive isolates, four were *ica* positive (2 *ica ADBC* positive, 2 *ica AD* positive) (Table I). Of the 18 biofilm negative isolates 14 turned biofilm positive (2 *ica AD* positive and 12 *ica* negative) and four remained biofilm negative (all *ica* negative) (Table II).

***Coagulase negative staphylococci*:**

Invasive isolates - The mean absorbance value in different culture conditions increased from 0.353±.03 (control) to 1.46±.06 (highest in presence of both glucose and NaCl). All nine (*S. haemolyticus*) biofilm negative isolates turned biofilm positive (5 *ica ADBC* positive, 2 *ica AD* positive and 2 *ica* negative) in presence of glucose/NaCl/ both (Tables I, II).

Colonizing isolates - The mean absorbance value in different culture conditions increased from 0.453±.04 (control) to 1.33±.10 (highest in presence of both glucose and NaCl). Of the 12 *S. epidermidis* biofilm positive isolates, 10 were *ica* positive (7 *ica ADBC* positive, 3 *ica AD* positive) and two were *ica* negative (Table I). Five of eight biofilm negative isolates turned biofilm positive (one *S. xylosus ica ADBC* positive, one *S. xylosus* and 2 *S. epidermidis ica AD* positive and 1 *S. epidermidis ica* negative) in presence of glucose/ NaCl/both but three isolates remained biofilm negative (Table II).

Commensal isolates - The mean absorbance value in different culture conditions increased from 0.301±.03 (control) to 0.755±.07 (highest in presence of both glucose and NaCl). Of the 9 biofilm positive *S. epidermidis* isolates, six were *ica* positive (4 *ica ADBC* positive, 2 *ica AD* positive). Of the 16 (12 *S. epidermidis,* 1 *S. saprophyticus)* biofilm negative isolates, 13 turned biofilm positive (1 *S. epidermidis* was *ica ADBC* positive, 1 *S. Saprophyticus* and 3 *S. epidermidis* were *ica AD* positive and 8 *ica* negative) in presence of glucose/NaCl/ both and three isolates remained biofilm negative (all *ica* negative) (Tables I, II).

The present study showed significant increase in biofilm production by clinical isolates of *Staphylococcus* when exposed to increasing concentration of glucose and sodium chloride. Increase was higher when NaCl and glucose both were supplemented in the medium. No significant biofilm formation has been reported if bacteria are grown in TSB alone irrespective of the fact that TSB already contains substantial amounts of glucose[5]. Supplementation of 0.2 per cent glucose in TSB is sufficient to induce a visible biofilm formation and a further increase in glucose concentration up to 1 per cent increases the biofilm formation significantly[7,9,12]. Rode *et al*[6] compared individual effects of glucose and NaCl with the combination of glucose plus NaCl. Strains showed highest biofilm

Table II. Biofilm negative *S. aureus* and CNS isolates turned biofilm positive in different culture conditions

Groups	*S. aureus*						Coagulase negative staphylococci						
	Biofilm –ve	Biofilm -ve isolates turned biofilm +ve					Remain -ve	Biofilm -ve	Biofilm -ve isolates turned biofilm +ve				Remain -ve
		A	B	C	D	E			A	B	C	D	E	
Invasive	17	6	11	14	15	15	2	9 Sh	6	6	8	9	9	0
Colonizing	8	3	4	5	5	5	3	5 Se	-	1	2	2	2	3
								3 Sx	2	3	3	3	3	0
Commensal	18	2	3	7	12	14	4	14 Se	3	5	7	12	12	2
								2 Ss	1	1	1	1	1	1
Total	43	11	18	26	32	34	9	33	12	16	21	27	27	6

Sh, *Staphylococcus haemolyticus*; Se, *Staphylococcus epidermidis*, Sx, *Staphylococcus xylosus*; Ss, *Staphylococcus saprophyticus*; TSB, trypticase soy broth; A, TSB alone; B, TSB+5% glucose; C, TSB+10% glucose; D, TSB+0.9% NaCl; E, TSB+5% glucose+0.9% NaCl

formation in the presence of glucose and NaCl rather than for each compound separately.

Expression of the *ica ADBC* operon is considered to be essential for the synthesis of polysaccharide intercellular adhesin (PIA), which mediates cell-to-cell adhesion[1]. Some biofilm producing isolates were found to be *ica* operon negative in our study, as has been reported earlier[4]. Cafiso *et al*[13] analyzed the transcriptional activity of *ica* operon genes in a sample of biofilm positive and biofilm negative staphylococcal isolates and concluded that biofilm production takes place only when *ica D* was co-expressed with *ica A*. *ica* independent mechanism for biofilm formation was first studied by Cucarella *et al*[14], who demonstrated that biofilm associated proteins (Bap) were involved in *ica* independent biofilm formation mechanism[15]. The production of PIA is subjected to on-off switching, and may be involved in phase-variation that might improve bacterial survival and growth under changing environmental conditions *in vivo*[16]. Environmental regulation may play an important role in biomaterial related disease. *ica*R is a strong negative regulator of the *ica* locus, as deletion of *ica*R augmented PIA production by nearly 10-fold and increased transcription of the *ica* locus by 100-fold[17]. The expression of this gene alone induces low enzymatic activity and the production of low amounts of polysaccharide. However, the simultaneous expression of *icaA* and *icaD* promotes a significant increase in N-acetylglucosaminyl transferase, with a consequent increase in the amount of polysaccharide, forming oligomers of 10-20 β-1,6-N-acetylglucosamine residues[17].

Several *ica* independent regulatory genes have also been reported. The *agr* gene has been shown to increase biofilm detachment and mutation of the system, leading to decreased biofilm growth[18,19]. Staphylococcal accessory regulator (*Sar*) gene regulates genes of cell wall-associated adherence factors increasing the *ica* transcription and PIA/poly-N-acetyl glucosamine (PNAG) production[20,21]. Additional components, such as accumulation-associated protein (Aap), DNA and RNA independently or in cooperation with the *ica* operon, have also been suggested to be important in CNS biofilms[22-24]. Bap has been shown to be involved in the initial attachment, intercellular adhesion and biofilm formation of *S. aureus*[14]. NaCl has been found to significantly induce more biofilm in methicillin susceptible *Staphylococcus aureus* (MSSA) than in methicillin resistant *Staphylococcus aureus* (MRSA) isolates[25]. NaCl is a known activator of *ica* transcription[26,27] and biofilm development in MSSA is *ica ADBC* dependent. Biofilm development in other isolates is primarily glucose induced, may be *ica* independent and involves a protein adhesion[28].

In conclusion, our findings show that increasing the concentration of glucose and sodium chloride enhances biofilm production capacity of pathogenic as well as non pathogenic staphylococcal isolates irrespective of presence or absence of *ica* operon.

Acknowledgment

The work was supported by a grant from Indian Council of Medical Research, New Delhi.

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