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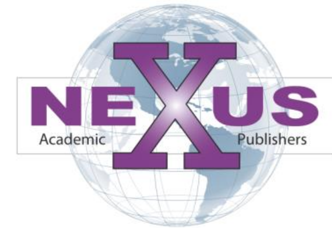

Research Article

Genotypic and Phenotypic Characterization of Clinical Isolates of *Staphylococcus aureus* for Biofilm Formation Ability

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ABSTRACT

The objective of the study was to characterize *Staphylococcus aureus* (*S. aureus*) isolated from human and animal clinical cases for their biofilm formation ability by genotypic and phenotypic methods. A total of 130 *S. aureus* strains isolated from human wound (n = 20), animal wound (n = 70) and animal mastitis (n = 40) cases were subjected to screening for 3 different biofilm associated genes (*bap*, *icaA* and *icaD*) and for phenotypic assessment for biofilm formation using Congo red agar, modified Congo red agar and microtitre plate assay. PCR assays were standardized for the detection of *bap*, *icaA* and *icaD* genes. The results indicated that *icaA* gene was present in 51.15% of the isolates and *bap* gene was present in 8.46% isolates. None of the isolates were positive for *icaD* gene. Human isolates (65%) had higher occurrence of *icaA* gene in comparison to animal isolates (49.09%). Dog wound isolates had higher occurrence of *bap* gene. Of the 3 methods used for phenotypic expression of biofilm by *S. aureus* isolates modified Congo red agar method showed 86.92% isolates to be positive, whereas by Congo red agar method only 63.07% *S. aureus* were found to be biofilm producer. Microtitre plate assay showed 75.38% *S. aureus* isolates to be biofilm producers. A good correlation was observed between genotypic and phenotypic biofilm formation ability of the isolates. *Bap* gene contained isolates showed higher biofilm producing ability compare to *icaA* gene harbored isolates.

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INTRODUCTION

Staphylococcus aureus is a Gram–positive bacterium and is an important human and animal pathogen. The *S. aureus* causes a wide variety of infections ranging from mild skin infections, to life–threatening diseases such as bacteremia. The pathogenesis of *S. aureus* is attributed to the combined effect of extracellular factors and toxins, together with the invasive properties of the strain such as adherence, biofilm formation, and resistance to phagocytosis. There is general agreement that biofilms are the basis for persistent or chronic bacterial infections (Costerton et al., 1999). The implication of biofilms in chronic infections has triggered an increasing interest in the organization of genes involved in biofilm formation (Caizza and O'Toole, 2005; Tormo et al., 2005). The *icaADBC* cluster, an operon present in *S. aureus* and *S. epidermidis*, participates in biofilm formation by encoding proteins involved in the synthesis of a biofilm matrix polysaccharide (Cucarella et al., 2004). The *ica* operon was first identified and studied most extensively in *S. epidermidis* and was later shown to be present in *S. aureus* (Gotz, 2002). Most *S. aureus* strains appear to contain the entire *ica* operon, although there are reports to the contrary.

IcaA and *icaD* genes have been reported to play a significant role in biofilm formation in *S. aureus* and *S. epidermidis* (Gotz, 2002). The *ica* locus has been detected in majority of the mastitic *S. aureus* isolates indicating its potential role as a virulence factor in the pathogenesis of mastitis in ruminants (Ciftci et al., 2009, Milanov et al., 2010). Little information is available regarding genotypic characterization of *S. aureus* of animal and human clinical origin with reference to intercellular adhesion genes and its association with phenotypic characters of Indian isolates (Vasudevan et al., 2003, Dhanawade et al., 2010).

More recently, Cucarella et al., (2001) identified a surface protein (Bap, Biofilm Associated Protein) implicated in *S. aureus* biofilm formation. Vautor et al., (2008) has reported that the bap protein is a member of proteins playing a role in biofilm formation in many bacteria and they share common structural features as they have a high molecular weight and contain a core domain of tandem

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repeats. *Bap* gene has been identified in a small proportion of *S. aureus* from bovine mastitis (Cucarella et al., 2001). Many studies have yielded negative results for the presence of bap gene in human and animal cases (Arciola et al., 2001; Vasudevan et al., 2003; Vancraeynest et al., 2004; Nitzsche et al., 2007, Vautor et al., 2008) indicating low prevalence of this gene. No information is available about Indian *S. aureus* isolates.

A number of methods have been developed for cultivation and quantification of biofilm, such as tube test, microtiter plate test, radiolabeling, microscopy, Congo red agar plate test, etc. (Deighton et al., 2001; Mathur et al., 2006; Agarwal et al., 2011). Nevertheless, the microtiter plate and Congo red agar method remains among the most frequently used assays for investigation of biofilm (Vasudevan et al., 2003; Knobloch et al., 2002).

Therefore, the present study was undertaken to characterize *Staphylococcus aureus* isolated from human and animal clinical cases for their biofilm formation ability by genotypic and phenotypic methods.

MATERIALS AND METHODS

Bacterial strains

The bacterial stains included in the study are listed in the table 1. Most of the strains (111) were isolated in an earlier study in our laboratory in the year 2013. Twenty–nine bovine mastitis strains isolated in the year 2005 were procured from the repository of the Division of Standardization, Indian Veterinary Research Institute, Izatnagar, India. All the strains were tested for their purity, morphological and biochemical characteristics and were maintained by periodical sub culturing in brain heart infusion (BHI) broth.

Table 1: *S. aureus* isolates used in the study

Sr. No.	Source of isolates	Type of clinical case	No. of isolates
1.	Cattle	Wound	18
2.	Cattle	Mastitis	40*
3.	Dog	Wound	48
4.	Horse	Wound	1
5.	Goat	Wound	3
6.	Human	Wound	20
Total			130

* 29 of these were isolated in year 2005

Primers

Primers used in the study for detection of *icaA*, *icaD* and *bap* genes are listed in table 2.

PCR Protocol

All the 130 *S. aureus* isolates were subjected to amplification of *icaA icaD* and *bap* genes. The reaction mix invariably consisted of 5 µl of bacterial DNA, 2.5 µl of 10x PCR buffer for Taq polymerase [100 mM Tris HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin], 1.5 µl of 2.5 mM dNTP, 10 pmol of each forward and reverse primers, and 1 U of Taq polymerase. The final volume of 25 µl was made up by using milli Q water. For *icaA* and *bap* genes an annealing temperature of 55ºC was used, whereas for the amplification of *icaD* gene annealing was done at 49ºC. The cycling conditions used were an initial denaturation of 5 min at 94ºC, 35 cycle of 45 sec denaturation at 94ºC, 45 sec annealing at optimum temperature and 45 sec extension at 72ºC. Appropriate positive and negative controls were added in each of the PCR run. The PCR products were confirmed by agarose gel (1%) electrophoresis.

Phenotypic Expression of Biofilm

Biofilm formation ability of all the *S. aureus* isolates was studied by microtitre plate method and Congo red binding assay and modified Congo red assay.

Microtitre Plate Assay

The test was performed using tryptone soy broth containing 1% glucose (TSB + 1% Glucose). The isolates were first inoculated in the TSB + 1% glucose and incubated for 24 h at 37ºC. From each individual culture, 20 µl of TSB+1% glucose broth were dispended in the wells of sterile 96–well flat bottomed microtitre plate (Greiner bio–one) and kept for incubation at 37ºC for 48 h under aerobic conditions. Each strain was inoculated into at least 8 wells. The control well contained only TSB+1% glucose without inoculation. After incubation of plates, unbound cells were removed by inversion of microtitre plate, followed by vigorous tapping on absorbent paper. After that, adhered cells were fixed at 80ºC for 30 min.

Adhered cells on the bottom and side of the wells were stained by addition of 220 µl of crystal violet (0.5%) for 15 min and excess stain was rinsed off. The stain was removed by exhausting washing with distilled water. The plates were then allowed to air dry. After drying, 220 µl of decolouring solution (ethanol 80% and acetone 20%) was added to each well for 15 min. The absorption of eluted stain was measured at 590 nm in ELISA reader. Data obtained from above experiments was subjected to statistical analysis as per standard procedure of Snedecor and Cochran (1989).

Detection of Biofilm Associated Genes by PCR

Table 2: Details of the primers used in this study

Sr. No.	Target gene	Sequence	Product Size	Reference
1.	*bap*	F: 5–AAAGAGCCACATAAACAACAAGAA–3' R: 5'–GTAGCCATAGCACGGAACATAG–3'	368 bp	Self designed
2.	*icaH*–1m *icaH*–7c	F: 5'–TATACCTTTCTTCGATGTCG–3' R: 5'–CTTTCGTTATAACAGGCAAG–3'	550 bp	Cucarella et al. (2004)
3.	*icaD*	F: 5'–AAACGTAAGAGAGGTGG–3' R: 5'–GGCAATATGATCAAGATAC–3'	381 bp	Dhanawade et al. (2010)

Congo red Agar Assay

For Congo red assay two different methods were used to prepare plates. In first, Congo agar plates were prepared by adding brain heart infusion broth– 52 g, sucrose– 36 g, Congo red dye– 0.8 g and 2% agar. Modified Congo red agar was performed as suggested by Mariana et al. (2009).

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Isolates producing weak black, black or very black colonies were considered as biofilm producer and isolates with red colonies were considered as non biofilm producers.

RESULTS

Occurrence of Biofilm Forming Genes among S. aureus Isolates by PCR

The biofilm forming ability of the *S. aureus* isolates was assessed by studying occurrence of 3 different biofilm associated genes *viz*., *icaA*, *icaD* and *bap*. PCR assay was standardized for all the 3 genes by empirical variation of annealing temperature (50º–60ºC), concentration of primers (10 pmol to 15 pmol), template volume (3µl to 5µl) and the cycling conditions. PCR under optimized conditions yielded the desired sized product.

A comparison of occurrence of the 3 biofilm associated genes among all the *S. aureus* isolates indicated that *icaA* gene was present in 51.15% of isolates, followed by *bap* 8.46% genes (Table 3, Figure 1 and 2). None of the isolates were positive for *icaD* gene. Human isolates (65%) had more occurrence of *icaA* gene in comparison to animal isolates (49.09%). However, none of the human isolates carried bap gene. Among different animal isolates, dog wound isolates had higher occurrence of the *bap* gene.

Phenotypic Characterization of Biofilm Ability of S. aureus

Biofilm production ability was assessed by microtitre plate assay (Table 4). Out of 130 isolates, 2 isolates (1 human, 1 mastitis) produced very strong biofilm, 11 isolates (1 cattle wound, 2 cattle mastitis, 7 dog, 1 human, 1 mastitis isolates) produced strong biofilm, 32 isolates (3 cattle wound, 11 dog, 1 human and 17 mastitis isolates) produced moderate biofilm, 53 isolates (12 cattle wound, 10 cattle mastitis, 17 dog, 1 each of horse and goat, 11 human) produced weak biofilm. Remaining 32 isolates did not produce any biofilm. Overall, 75.38% isolates were adjusted as biofilm producers.

Biofilm production ability was also assessed by Congo red method (Table 5). Out of 130 isolates, 4 isolates (2 human and 2 mastitis isolates) produced very black colonies, 19 isolates (2 human, 2 cattle wound, 5 mastitis, 2 goat and 8 dog wound isolates) produced black colonies, and 59 (6 human, 23 dog, 6 cattle wound, 22 cattle mastitis, 1 goat and 1 horse wound) produced weak black colonies. Remaining 48 isolates produced red colonies, indicating them to be non biofilm producers.

Table 3: Prevalence of biofilm associated genes in *S. aureus* isolates.

Sr. No.	Source of isolates	Type of sample	No. of isolates tested	Positive for *bap* gene	Positive for *icaA* gene	Positive for *icaD* gene
A.	Animal					
1.	Cattle	Wound	18	2 (11.11%)	8 (44.4%)	0
2.	Cattle	Mastitis	40	1 (2.5%)	26 (65%)	0
3.	Dog	Wound	48	8 (16%)	18 (37.5)	0
4.	Horse	Wound	1	0	1 (100%)	0
5.	Goat	Wound	3	0	1 (33%)	0
Sub total (A)			110	11(10%)	54 (49.09%)	0
B.	Human					
1.		Wound	20	0	13(65%)	0
Grand total (A+B)			130	11(8.46%)	67(51.15%)	0

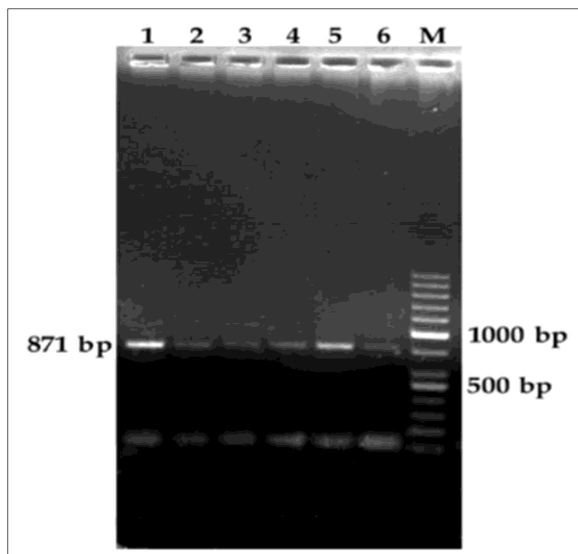

Figure 1: Results of PCR for *bap* gene of *S. aureus* isolates; Lane 1: *S. aureus* ASM–23; Lane 2: *S. aureus* ASW–25; Lane 3: *S. aureus* ASW–37; Lane 4: *S. aureus* ASW–39; Lane 5: *S. aureus* ASW–43; Lane 6: *S. aureus* ASW–45; Lane M: Marker

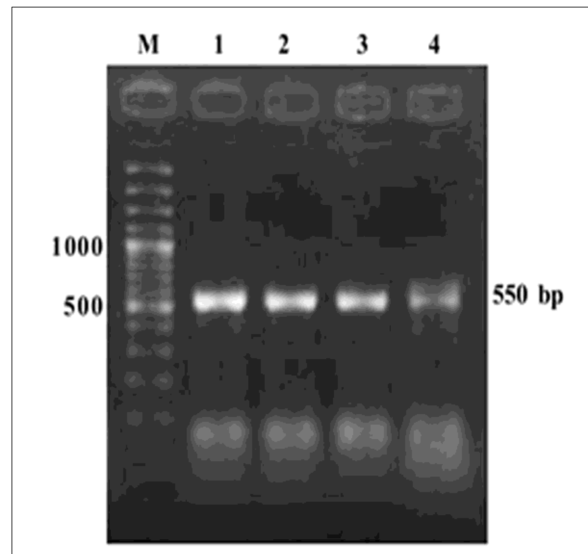

Figure 2: Results of PCR for *icaA* gene of *S. aureus* isolates; Lane M: Marker; Lane 1: *S. aureus* IVRI–42; Lane 2: *S. aureus* ASW–24; Lane 3: *S. aureus* IVRI–D7; Lane 4: *S. aureus* HS–12

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Table 4: Biofilm formation ability of *S. aureus* isolates by microtitre plate method

Sr. No.	Source of isolate	No of isolates tested	Non producer	Weak	Moderate	Strong	Very strong	Over all biofilm producer
1.	Cattle wound	18	2	12	3	1	–	16 (88.88%)
2.	Cattle mastitis	40	10	10	17	2	1	30 (75%)
3.	Dog wound	48	13	17	11	7	–	35 (72.91%)
4.	Horse wound	1	–	1	–	–	–	(100%)
5.	Goat wound	3	1	2	–	–	–	2 (66.66%)
6.	Human wound	20	6	11	1	1	1	14 (70%)
	Total	130	32	53	32	11	2	98 (75.38%)

Table 5: Biofilm formation ability of *S. aureus* isolates by Congo red method

Sr. No.	Source of isolates	Type of colonies Red	Weak black	Black	Very black	Total positive*
1.	Cattle wound	10	6	2	–	8 (44.44%)
2.	Cattle mastitis	11	22	5	2	29 (72.5%)
3.	Dog wound	17	23	8		31 (64.58%)
4.	Horse wound	–	1	–	–	1 (100%)
5.	Goat wound	–	1	2	–	3 (100%)
6.	Human wound	10	6	2	2	10 (50%)
	Total	48 (36.92%)	59 (45.38%)	19 (14.61%)	4 (3.07%)	82 (63.07%)

*Aggregate of isolates producing weak black, black and very black colonies

Table 6: Biofilm formation ability of *S. aureus* isolates by modified Congo red method

Sl. No.	Source of isolates	Type of colonies Red	Weak black	Black	Very black	Total positive*
1.	Cattle wound	5	10	3	–	13 (72.22%)
2.	Cattle mastitis	6	27	6	1	33 (82.5%)
3.	Dog wound	6	29	11	2	42 (87.50%)
4.	Horse wound	–	1	–	–	1 (100%)
5.	Goat wound	–	1	2	–	3 (100%)
6.	Human wound	–	14	4	2	20 (100%)
	Total	17 (13.07%)	82 (63.07%)	26 (20%)	5 (3.84%)	113 (86.92%)

*Aggregate of isolates producing weak black, black and very black colonies

Table 7: Comparison of phenotypic and genotypic biofilm formation ability of *S. aureus* isolates

Sr. No.	Pheotypic characteristic	ica A gene No. of positive isolates	No. of negative isolates	*Bap* gene No. of positive isolates	No. of negative isolates
1.	Phenotypically positive* (119)	67 (56.30%)	53 (44.53%)	11 (9.24%)	101 (84.87%)
2.	Phenotypically negative (11)	0	11 (100%)	0	11 (100%)

*Positive by any of the 3 methods used

On modified Congo red agar, out of 130 isolates, 5 isolates produced very black colonies, 26 isolates produced black colonies, 82 isolates produced weak black colonies and 17 isolates showed red colonies (Table 6).

Comparison of genotypic and phenotypic biofilm characteristics of the isolates revealed that the all the 11 phenotypically negative isolates were also negative for *icaA* and *bap* genes (Table 7). Of the 119 phenotypically positive isolates 56.30% isolates demonstrated the presence of *icaA* gene, whereas 9.24% isolates were positive for *bap* gene

DISCUSSION

The *S. aureus* has been shown to posses the capability to form biofilm (Costerton et al., 1999; Branda et al., 2005; Heilmann et al., 1997; Caizza and O'Toole, 2005; Tormo et al., 2005; Cucarella et al., 2001; Vasudevan et al., 2003, Dhanawade et al., 2010; Vautor et al., 2008), which help bacteria to adhare to an inert or living surface (Costerton et al., 1999) and is able to adhere and form biofilm consequently causing severe morbidity and infection (Sheagren, 1984; Waldvogel et al., 1995). The ability to produce biofilm is also the most important reason for eradication of infection and recurrent infections of mammary glands in bovine mastitis caused by *S. aureus* (Melchior et al., 2006b). Biofilm production enables adhesion of bacteria to the epithelium of mammary glands. It also facilitates persistence of micro–organisms in the host tissue by protecting the bacterial cells against the mechanisms of the host defense (Melchior et al., 2006a, 2007). Production of biofilm requires the presence of the intracellular adhesion locus gene cluster icaADBC (Cramton et al., 1999) and strains harboring the icaADBC cluster are potential biofilm producers. In addition, biofilm– associated protein (*Bap*) is also for the primary attachment and cells' accumulation (Cucarella et al., 2001; Lasa and Penadés, 2006).

In our study, we examined the ability of biofilm production in *S. aureus* by detecting *icaA* and *icaD* genes in all

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isolates. We tested a total of 130 isolates for the presence of *icaA* and *icaD* genes. Of the 110 animal isolates 54 (49.09%) isolates were positive for *icaA* gene, but no isolate was positive for *icaD* gene. The distribution of *icaA* gene among different animal isolates was 44.4% (8/18) in cattle wound, 65% (26/40) in cattle mastitis, 37.5% (18/48) in dog wound, 100% (1/1) in horse wound, 33% (1/3) in goat wound. Earlier workers have reported variation in the occurrence of these genes. Melchior et al. (2009) found 74 out of 99 strains isolated from mastitis in Netherlands to be positive. Cifti and coworkers examined the group of 59 isolates from mastitis and found only 16 *icaA* positive stains, 38 strains harboured the *icaD* gene and 15 of them contained both genes (Cifti et al., 2009). Among the group of 102 *S. aureus* mastitis isolates from India, only 36 revealed the presence of both genes (Dhanawade et al., 2010). On the other hand, several authors showed presence of the *ica* locus genes in all *S. aureus* clinical isolates analyzed in their studies (Fowler et al., 2001; Rohde et al., 2001; Knobloch et al., 2002; Asthan and Shamsudin, 2011, Szweda et al., 2012). These variations could be due to circulations of different clones of *S. aureus* in different regions.

In the case of strains isolated from human wound infection, 13 isolates out of 20 were *icaA* positive. But none of the isolates possessed *icaD* gene. In contrast, 36/46 isolates from auricular infections in Tunisie were *icaA* and *icaD* positive (Zmantar et al., 2010), while Grinholc and Coworkers were not able to detect the presence of *icaD* gene in case of 27 strains among the tested group of 302 clinical MRSA isolates, whereas all of them harbored the *icaA* gene (Grinholc et al., 2007). Rohde et al. (2007) found that all *S. aureus* had the *icaA* gene. On the other hand, Arciola et al. (2001) suggested that 60.86% strains of *S. aureus* had *icaA* and icaD. It has been demonstrated that most of the *S. aureus* strains contain the entire ica operon (Cramton et al., 1999; Arciola et al., 2001a).

We also studied the occurrence of *bap* gene among the *Staph aureus* isolates. PCR assay was successfully standardized employing self–designed primers. Eleven (8.46%) isolates out of 130 showed the presence of *bap* gene, of which 2 were from cattle wound, 1 from cattle mastitis and 8 from dog wound samples. *Bap* gene was not present in any of the goat, horse and human isolates. These results are in agreement with the previous reports on *S. aureus* by Cucarella et al. (2001) who found only 5% isolates positive for *bap* gene. Szweda et al. (2012) did not report *bap* gene in any of isolates in his study in Poland. Similar, results were found in *S. aureus* isolates of different origin, such as human, sheep, goat, bovine, pig, poultry, horse and rabbit (Vancraeynest et al., 2004; Nitzsche et al., 2007; Vautor et al., 2008; Melchior et al., 2009), Vantor and cowoekers (2008), hypothesized that the *bap* gene has not spread among the *S. aureus* isolates of animal and human origin and its prevalence is very low.

Interestingly, in contrast *bapA* gene has shown to be high conserved in *Salmonella* (Lasata et al., 2006; Lassa and Penades, 2006). But in *S. aureus*, only some isolates (5%) from clinical mastitis cases and other clinical cases in animal and human are reported to carry *bap* gene (Cucarella et al., 2001). The low prevalence of *bap* gene in *S. aureus* may be because of either the gene is recently acquired in the pathogenecity island SaPIbov2, a mobile genetic element or the horizontal transfer is not easy (Vautor et al., 2008).

Phenotypic expression of biofilm formation was studied by Congo red agar assay, modified Congo red assay and microtiter method. In present study, out of 130 strains of *S. aureus* isolates from animal and human clinical cases, 63.07% strains were considered biofilm producer by Congo red agar method. In which, 3.07% isolates produced very black colonies, 14.61% produced black colonies, 45.38% produced weak black colonies and remaining 63.07% produced red colonies. Previous work on biofilm formation carried out by Krukowski et al. (2008), reported 42% biofilm producing *S. aureus* by CRA method. In another study, 42% isolates were found to be biofilm producer (Dziekiewicz – Mrugasiewicz et al., 2008). Szweda et al. (2012) found 57% positive isolates by CRA method.

Modified Congo red method was also used to study the biofilm production ability of *S. aureus* (Mariana et al., 2009). In this study, we found 86.92% isolates to be positive for biofilm production. Similar findings have been reported by Atshan et al. (2012).

The microtiter plate method remains among the most frequently used assays for investigation of biofilm. In this study, we also optimized microtitre plate method and used it for testing all the 130 *Staph aureus* isolates for biofilm formation ability. Of these 130 isolates, 75.38% were found to be slime producers by microtitre plate assay. Gundogan et al. (2006) found that 58 out of 110 *S. aureus* strains were slime producers by this method. Furthermore, Vasudevan et al. (2003) demonstrated that 32 of 35 *S. aureus* isolates were slime positive and Zmantar et al. (2010) found that 26 out of 46 strains of *S. aureus* (56.5%) were slime producers. However, Atshan et al. (2012) found 100% isolates to be biofilm producers.

Comparison of genotypic and phenotypic biofilm characteristics of the isolates revealed that absence of either of 2 genes studied (*icaA* and *bap*) clearly indicated that the *S, aureus* isolates were negative for biofilm production. Occurrence of these 2 genes was variable among the phenotypically positive *S. aureus* isolates, indicating that there are other factors involved in biofilm formation. However, the *bap* gene containing isolates showed higher biofilm producing ability in both, microtitre plate and Congo red assays (result not shown). We conclude that the presence of bap gene in *S. aureus* isolate will definitely indicate high biofilm production ability.

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