

Optimum cultural conditions to achieve the best biofilm formation by *Staphylococcus aureus*

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Abstract

The aim of this study was to investigate the influences of different broth culture media supplemented with glucose, on the biofilm formation and *ica* expression of *Staphylococcus aureus*. The phenotypic ability to adhere to a polystyrene surface and to produce slime layer were evaluated using microtiter plate test (MtP) and Congo red tube test, respectively. Using PCR, the presence of *ica* locus in *S. aureus* strains was confirmed and subsequently, quantitative real-time RT-PCR was performed to investigate transcription of *icaA* in various media including Tryptic soy broth (TSB), Brain-heart infusion broth (BHIB), (Nutrient broth) NB and (Muller-Hinton broth) MHB contained 0, 0.25, 0.5, 1 and 2% glucose. Our results showed that although all of the studied strains adhered to the wells of polystyrene microtiter plates, the optimum rate of biofilm formation was observed for TSB medium contained 1% glucose, but biofilm formation was not significantly different in NB, MHB and BHIB media. Supplementation of all media with 1% glucose led to the highest production of biofilm formation and in all of media transcription of *icaA* was increased with glucose addition to one present. The results of the present study indicated that TSB medium supplemented with 1% glucose was the most appropriate medium for evaluation of biofilm formation by *S. aureus* isolates.

Key Points

- *S. aureus* is an important human, animal and food-borne pathogen able to form biofilms.
- Addition of 1% glucose to bacterial culture media increases biofilm formation as well as *ica* expression.
- Optimum rate of biofilm formation was occurred for TSB medium contained 1% glucose.

Introduction

Biofilms is an adherent community of bacteria that irreversibly attached to a substratum or to each other. Biofilm infections, such as pneumonia in cystic fibrosis patients, chronic wounds, chronic otitis media and implant- and catheter-associated infections, affect millions of people in the developed world each year and many deaths occur as a consequence (Bjarnsholt, 2013; Khan et al., 2021). Structural analysis of biofilms often reveals that bacterial biofilms are often surrounded by an extracellular polymeric substance (EPS) (Szweda. 2012). The gene expression pattern of bacteria in a biofilm is different from planktonic cells. This difference in gene expression along with other characteristics including physical barrier of exopolysaccharides can cause increased tolerance against antibiotics, disinfectants, and host immune mechanisms in biofilms (Lewis, 2007). *S. aureus* is one of the most important bacterial species which can form biofilms on biotic and abiotic surfaces (Sharifi et al., 2018; Sharifi et al., 2021; Yu et al., 2012). For instance, *S. aureus* biofilms may be involved in infections of central venous catheters, urinary catheters, prosthetic heart valves, orthopedic implants, and dialysis catheters. *S. aureus* EPS is mainly composed of poly-N-acetylglucosamine polymer which is encoded by *icaADBC* operon (Francois et al., 2000; Wang et al., 2017; Sharifi et al., 2018).

Several phenotypic and genotypic methods are available for the detection of staphylococcal biofilms. Phenotypic methods such as MtP and Congo red agar (CRA) tests measure biofilm density and production of slime layer, respectively, while, genotypic methods like PCR, determine biofilm related genes e.g. those encoded microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Christensen et al., 1985; Freeman et al., 1989). MtP test uses 96-well microtiter plates to measure the optical density (OD) values of stained bacterial biofilms. This method gives a quantitative result (Christensen et al., 1985).

In CRA method, bacteria are cultured on Congo red agar and incubated; thereafter, the color of colonies indicates their ability to produce slime layer. Slime-forming strains appear as black colonies. whereas, non-slime-forming strains stain red (Freeman et al., 1989). In addition, the MSCRAMMs mediate *S. aureus* attachment to different surfaces (Seo et al., 2008). Actually, intercellular signaling between bacteria, which is also known as quorum sensing, has been shown to be involved in the development of biofilm by *S. aureus*. Quorum-sensing occurs based on the synthesis and secretion of small hormone-like molecules (autoinducers). These molecules can bind to cognate receptors and contribute to biofilm formation. Quorum sensing in these bacteria is encoded by an accessory gene regulator (*agr*) locus. Activation of *agr* system can affect *S. aureus* biofilm formation through down-regulation of MSCRAMMs as well as production of a protease (Yarwood et al., 2004). It has been described that bacterial species, attachment surface and surrounding medium are three important factors involved in the formation of biofilms (Van Houdt and Michiels, 2010). The ability of each strain to adhere and form biofilm is very different in various culture media and environmental conditions (Van Houdt and Michiels, 2010), However, environmental factors such as pH, temperature, osmolarity, O₂ levels, nutrient composition and the presence of other bacteria can affect the ability of a microorganism to produce biofilm (Di Bonaventura et al., 2007). In this case, it has been shown in a study that glucose may repress *agr* system by lowering pH which results in increased biofilm formation (Regassa et al., 1992). Thus, the present study was conducted to introduce an appropriate bacterial culture medium with optimum glucose concentration for examination of the ability of biofilm formation and *ica* transcription by *S. aureus* strains.

Materials And Methods

Bacterial strains, culture media and growth conditions

S. aureus ATCC 25923 was used as a reference biofilm producer strain in all of the experiments. Besides, two *S. aureus* strains isolated from tracheal tube samples of patients admitted to the ICU of a hospital in Hamedan (west of Iran) were also included in the present study. These bacterial isolates were identified by culture characteristics and biochemical tests and their identity was also confirmed by a species-specific PCR assay which investigated *femA* gene of *S. aureus* (Kobayashi et al., 1994). Meanwhile, *S. epidermidis* ATCC 12228 was used as a non-biofilm producer control in the assays. All culture media were obtained from Merck (Darmstadt, Germany) and the bacteria were cultured under aerobic conditions at 37°C for 24 h.

Microtiter Plate Assay

All *S. aureus* isolates were examined for the ability of biofilm formation using a previously described microtiter plate assay (Dhanawade et al., 2010). Briefly, 50 µl of an overnight culture of each isolate was added into 96-well microplates (SPL, Korea) containing 50 µl of each of the Nutrient broth (NB), Tryptic Soy broth (TSB), Mueller-Hinton broth (MHB), and Brain-heart infusion broth (BHIB) media supplemented with 0.25%, 0.5%, 1% and 2% glucose (Merck, Germany). NB, TSB, MHB and BHIB media without glucose were also used as control group. The microplates were aerobically incubated at 37°C for 24 hours. The supernatants were then discarded and microplates were washed twice with phosphate-buffered saline (PBS). The plates were left to dry for an overnight and stained with 200 µl of 0.1% Safranin dye for 15 min followed by three times washing with 300 µl of PBS and air-dried for an overnight. Afterwards, 100 µl of ethanol-acetic acid (95:5 V/V) was added to each well to dissolve bounded Safranin in adherent bacterial cells. Finally, optical density values were measured at 490 nm by a plate reader. The experiment was performed in triplicates. The biofilm-producer *S. aureus* ATCC 25923 and the biofilm non-producer *S. epidermidis* ATCC 12228 were used as control strains (He et al., 2014).

Slime Assay

Congo red tube test was performed to evaluate the ability of slime production in different media supplemented with various glucose concentrations. To do this, a single colony from an overnight culture was inoculated into 10 ml of each of NB, TSB, MHB and BHIB media contained 0.04% Congo red dye and supplemented with 0.25, 0.5, 1 and 2% glucose. NB, TSB, MHB and BHIB media without glucose were used as control group. The culture tubes were incubated on a shaker (100 rpm) at 37°C for 24 hours. The cultured media were then categorized into three groups based on their colors: black, weak black and red. As exopolysaccharides directly react with Congo red and produce black color, the level of black color is considered as the amount of exopolysaccharides or polysaccharide intercellular adhesins (PIA) (Christensen et al., 1982). Therefore, the black and red media were considered to be related to normal slime producing and non-slime producing isolates, respectively.

RNA Extraction and cDNA Synthesis

To evaluate transcription of *icaA* gene, the presence of its encoding DNA was first investigated in *S. aureus* isolates and the reference strain by a PCR assay using previously introduced primers (Yazdani et al., 2006). The sequence of forward primer was 5'-TATTCAATTTACAGTCGCAC-3' and of reverse primer was 5'-GATTCTCTCCCTCTCTGCCA-3'. These primers amplify a 407 bp DNA fragment of *icaAD* gene based on a published sequence (AF086783). A PCR product was sequenced to confirm PCR specificity. For removing DNA contamination, the extracted RNA was treated with RNase-free DNase I (Thermo Scientific, USA). The quality and quantity of the extracted RNA were determined by agarose gel electrophoresis and confirmed by measuring the absorbance at 260 nm using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Extracted RNAs were stored at -70°C for next experiments. Then, the purified RNA was converted to cDNA according to the

manufacturer's instructions (cDNA Synthesis Kit, Takara, Japan), and stored at -20°C to use as the template for real time RT-PCR.

Relative quantitative real-time RT-PCR

SYBR Green real-time PCR Master Mix (Amplicon, Denmark) was used for real-time RT-PCR, according to the manufacturer's instructions. The primers (Takapouzist, Iran) used are listed in Table 1. The reactions were conducted in a Corbett Life Science Rotor-Gene 6000 Cycler (Qiagen, Germany) and *16S rRNA* housekeeping gene was considered as an internal control to normalize the expression levels of *icaA*. The amplification proceeded as follows: denaturation at 95°C for 10 min and then 40 cycles including denaturation at 95°C for 30 sec, annealing at 53°C (for *icaA*) and 59°C (for *16S rRNA*) for 30 sec, and 72°C for 30 sec. A negative control was included in each run. All the samples were analyzed in triplicate and finally, relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 1
Primers used for the quantitative real-time RT-PCR assay.

Genes	Sequence (5'-3')	Annealing temperature	Reference
<i>icaA</i>	GGAAGTTCTGATAATACTGCTG	53°C	(Harapanahalli et al., 2015)
	GATGCTTGTTTGATTCCCTC		
<i>16S rRNA</i>	AGCCGACCTGAGAGGGTGA	59°C	(Koprivnjak et al., 2006)
	TCTGGACCGTGTCTCAGTTCC		

Statistical Analysis

One-way ANOVA was carried out to compare OD values obtained from three independent experiments using SPSS software. The mean difference was considered significant at $P < 0.05$.

Results

Bacterial isolates

All of the isolates were genotypically confirmed as *S. aureus*. Figure 1 depicts the results of electrophoresis of the species-specific PCR.

Microtiter Plate Assay

The ability of biofilm formation was investigated by measuring OD values of the examined isolates using MtP assay. In all of the tested media, the biofilm formation rate of *S. epidermidis* 12228 was significantly lower than *S. aureus* 25923 and the other two isolates. However, the results revealed that no significant difference was observed in the rate of biofilm formation between *S. aureus* 25923 and the others. On the other hand, the results showed that the highest and lowest rates of biofilm formation occurred when

bacteria were cultured in TSB and NB, respectively. The biofilm formation patterns for two clinical isolates were similar to what was seen for *S. aureus* 25923 (data are not shown). Addition of glucose from 0 to 1% in all media also showed a significant positive effect on the biofilm formation of *S. aureus* ($P < 0.05$) and the most biofilm formation was observed in media with 1% glucose. However, increasing of glucose percentage up to 2 percent did not promote biofilm formation. Some reactions of biofilm formation in MtP test are shown in the Fig. 2.

Slime Assay

In Congo red tube test, *S. aureus* 25923 and the other two clinical isolates (biofilm producer strains) changed the color of MHB, NB and TSB media from red to reddish grey after the incubation period. However, *S. epidermidis* 12228 did not change the base red color of all of the examined media. In other words, no significant difference was observed among these media in the test. Addition of glucose did not show any visible effect on the tested media (Fig. 3).

Real time-PCR for evaluation of *icaA* gene expression

The results of the conventional PCR showed that *icaA* was present in all three tested strains (two clinical isolates and *S. aureus* ATCC 25923). Sequencing of this PCR product confirmed the accuracy of PCR assay. The sequence obtained for *icaA* amplicon was 99% identical to the corresponding GenBank sequence (accession number WP_031785277.1). The results of real time-PCR indicated that expression of *icaA* was increased by addition of glucose to 1% in TSB, MHB, NB and BHIB media. Figure 4 depicts the results of real time PCR for *icaA* expression.

Discussion

The chemical components of growth media and their supplements such as NaCl, ethanol, glucose and sub-inhibitory concentrations of antibiotics, strongly affect biofilm development (Rohde et al., 2001). In particular, the presence of glucose in the growth medium has been reported to enhance biofilm formation (Stepanović et al., 2007). Various methods have been introduced to measure the biofilm-forming capability of *S. aureus* isolates, among which, MtP is one of the most common methods in bacteriology laboratory. In this technique, TSB medium containing various glucose concentrations is often used to study the adherence ability of *S. aureus* to 96-well polystyrene tissue culture plates (Dhanawade et al., 2010).

In the present study, the ability of biofilm formation by *S. aureus* strains was investigated in four bacterial culture media (NB, TSB, MHB and BHIB) containing various glucose supplements (0, 0.25, 0.5, 1 and 2%) using MtP assay. The results showed that among all of the tested broth media, TSB was the most suitable medium to examine the ability of biofilm formation. However, some studies reported that bacterial biofilm formation in BHIB is sometimes occurred better than TSB (Kennedy and O'Gara, 2004; Mathur et al., 2006; Stepanović et al., 2007). Nevertheless, it has also been shown that some staphylococcal strains produce greater biofilm in TSB compared to BHIB (Knobloch et al., 2002). Moreover, researchers commonly use TSB with various glucose supplements for evaluation of biofilm

formation by *S. aureus* strains (Kennedy and O'Gara, 2004). It has been reported that addition of glucose to culture media lead to the increase of the ability of staphylococci biofilm formation (Knobloch et al., 2002; Mathur et al., 2006). In agreement with the previous findings, the present study showed significant increase in *S. aureus* biofilm production when the bacterium exposed to the increasing concentrations of glucose. The best concentration of glucose for biofilm formation of *S. aureus* on polystyrene surfaces in all of the tested culture media was 1%.

In addition, Congo red tube test and real time-PCR were used to detect extracellular exopolysaccharide and demonstrate *icaA* expression, respectively. RT-PCR results showed that *icaA* was expressed in all of the media with different glucose concentrations but the most expression occurred when one percent glucose was added to the medium ($P < 0.05$). Within the *icaADBC* operon, the *icaA* gene encodes the enzyme N-acetylglucosaminyl transferase, capable to catalyse the synthesis of poly-N-acetylglucosamine polymer which is called PIA and is important for cell to cell adhesion and accumulation (Gerke et al., 1998). The *icaADBC* and products of the *ica* locus [*icaR* (regulatory) and *icaADBC* (biosynthetic) genes] have been demonstrated to be necessary for biofilm formation (Croes et al., 2009). Consequently, in the present study, the expression of *icaA* locus was evaluated in different culture media supplemented with 0, 0.25, 0.5, 1 and 2% glucose. The results revealed that among all culture media (TSB, MHB, NB and BHIB) the most *icaA* expression level was detected in 1% glucose addition. In addition, the color of Congo red tube culture was not changed was not changed by glucose addition, indicating that this medium is not suitable for measurement of precise amount of PIA or measurement of *icaADBC* operon expression.

The results of the present study revealed that in fact, glucose increases the ability of biofilm formation by promoting *icaA* transcription and subsequently, PIA production. Hence, additional *ica*-independent factors may be involved in increasing of phenotypic biofilm formation by staphylococci. For instance, it was reported that Agr system is down-regulated by addition of glucose (Regassa et al., 1992), and *S. aureus* agr mutants also show enhanced biofilm formation (Vuong et al., 2000). Therefore, glucose-induced promotion of biofilm formation may be related to a suppression mechanism of agr. Using phenotypic analysis of wild type and mutant strains of *S. aureus*, Lim *et al* also described that *rbf* gene is another gene that mediates the biofilm formation of *S. aureus* at the multicellular aggregation stage in an *ica* independent manner (Lim et al., 2004), suggesting that the genetic basis of biofilm formation in staphylococci is multifactorial, and much remains to be explored.

In conclusions, the present study suggested that among various examined media, TSB supplemented with 1% glucose was the most appropriate medium for evaluation of biofilm formation of *S. aureus* strains. However, further studies are necessary to elucidate the precise mechanisms of increasing *S. aureus* biofilm formation following glucose induction to culture media.

Abbreviations

MtP: microtiter plate test; TSB: Tryptic soy broth; BHIB: Brain-heart infusion broth; NB: Nutrient broth; MHB: Muller-Hinton broth; EPS: Extracellular polymeric substance; CRA: Congo red agar; MSCRAMMs:

Microbial surface components recognizing adhesive matrix molecules; OD: Optical density; agr: Accessory gene regulator; PCR: Polymerase chain reaction

Declarations

Ethics approval and consent to participate

This research was approved by the Ethics Committee for Research at Hamedan University of Medical Sciences with ethical approval No IR.UMSHA.REC.1395.145. In addition, informed consent form was obtained from all of the patients.

All experiments and methods were performed in accordance with relevant guidelines and regulations. All experimental protocols were approved by a named institutional/licencing committee. Tracheal tube samples experiments (and relevant protocols) were approved by the Ethics Committee for Research at Hamedan University of Medical Sciences with ethical approval No IR.UMSHA.REC.1395.145.

Availability of data and material

The datasets generated during and/or analysed during the present study are available from the corresponding author [AM] on reasonable request.

Consent for publication

All the authors listed have approved the publication of this manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AS performed the laboratory experiments and wrote the manuscript. AM designed the research, and edited the manuscript. PM and TZS provided scientific consultations, and edited the manuscript.

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Figures

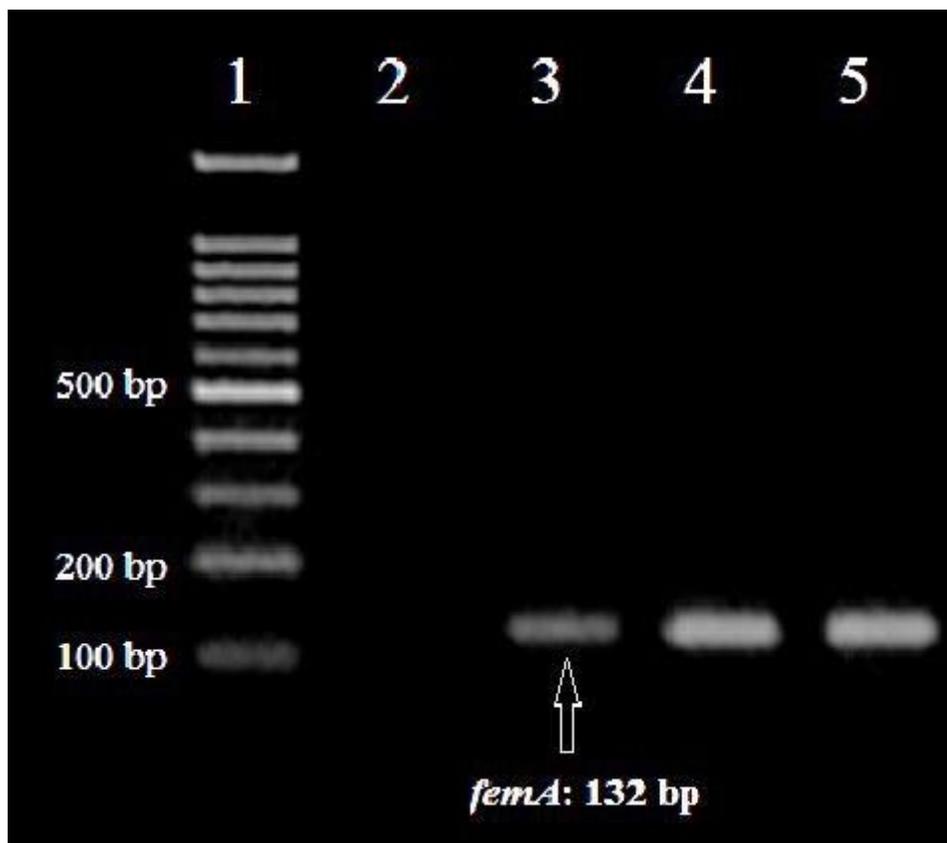


Figure 1

Agarose gel electrophoresis of species-specific PCR. Lane 1: 100 bp DNA ladder, lane 2: negative control (no template DNA), lane 3: positive control for *femA* gene (*S. aureus* ATCC 25923), lanes 4 and 5: PCR amplicons obtained from the isolates.

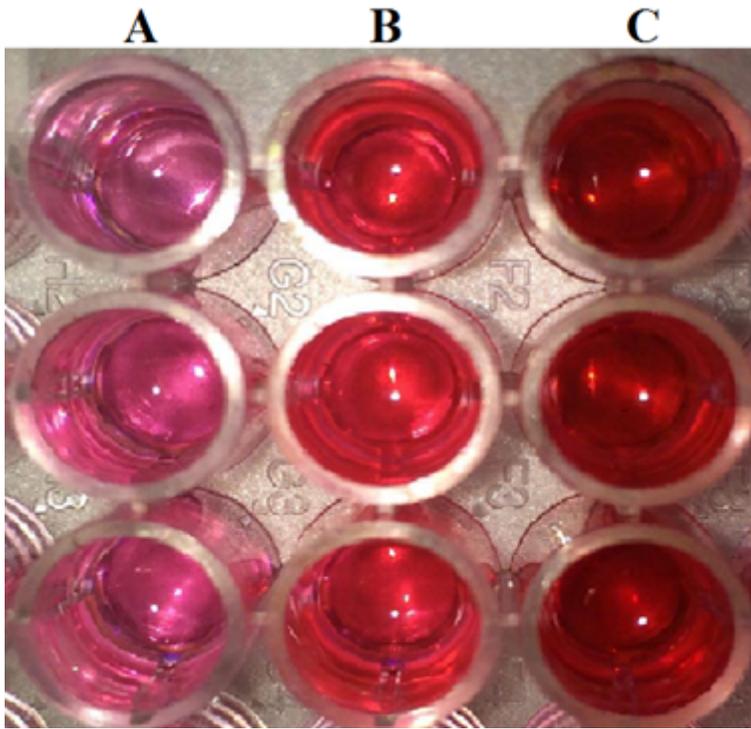


Figure 2

Measurement of the ability of biofilm formation using microtiter plate assay (MtP). Lanes A to C indicate the reaction of weak, moderate and strong biofilm producers, respectively.

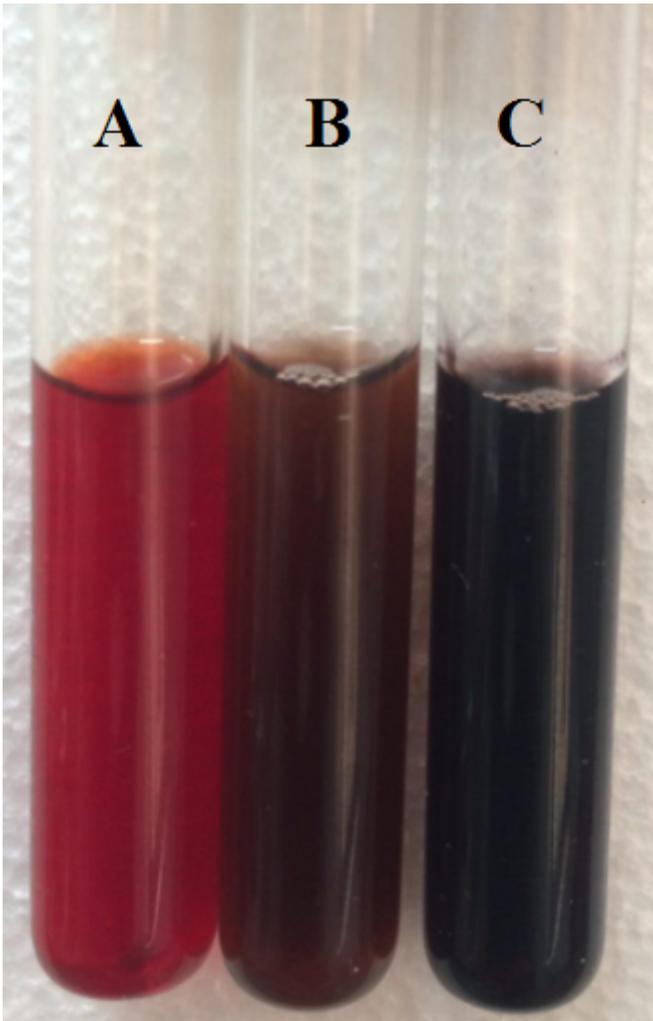


Figure 3

Screening of slime (PIA) producers by tube test. A: negative slime producer (*S. epidermidis* ATCC 12228, red pigments), B: moderate slime producer, and C: positive slime producer (*S. aureus* ATCC 25923, black pigments).

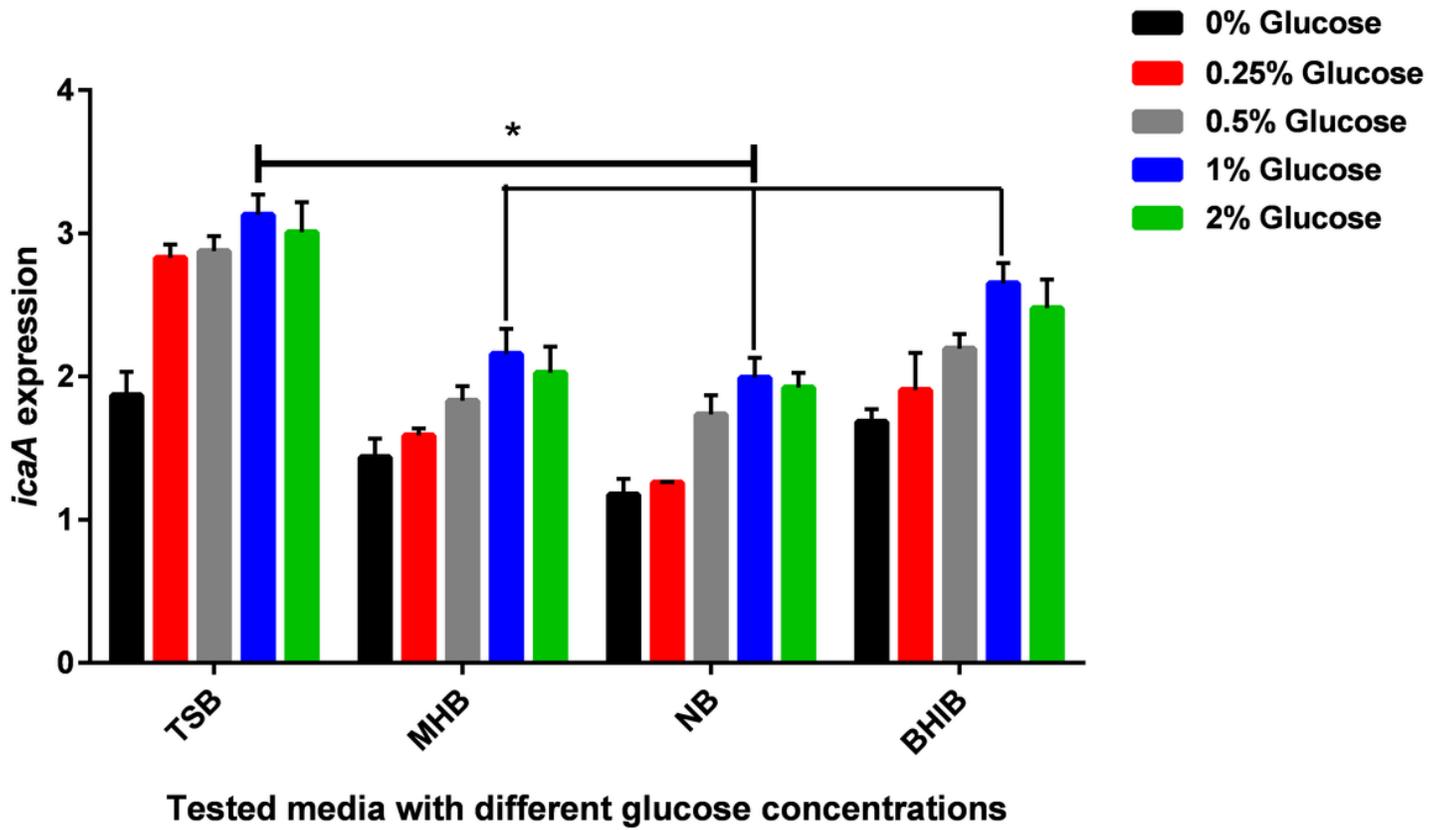


Figure 4

The *icaA* expression in various media contained 0, 0.25, 0.5, 1 and 2% glucose. The maximum *icaA* expression has occurred in TSB supplemented with 1% glucose. The error bars represent the standard deviation of three replicates (*: $P < 0.05$).