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Optimum culture conditions for achieving enhanced biofilm formation and high-level *icaA* transcription in *Staphylococcus aureus*

Aram Sharifi [1], Abdolmajid Mohammadzadeh [2]*, Pezhman Mahmoodi [2], Taghi Zahraei Salehi [3]

1. Department of Animal Science, Faculty of Agriculture, University of Kurdistan, Sanandaj, Kurdistan, Iran
2. Department of Pathobiology, Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamedan, Iran
3. Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

* Correspondence: Abdolmajid Mohammadzadeh. Department of Pathobiology, Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamedan, Iran. Tel: +988131406013; Email: mohammadzadeh@basu.ac.ir

Abstract

Background: Biofilm formation in *Staphylococcus aureus* (*S. aureus*), mediated by the *ica* operon, is a key virulence factor. This study examined how different glucose-supplemented broth culture media influence biofilm production and *ica* gene expression in *S. aureus*.

Methods: The phenotypic ability to adhere to a polystyrene surface and produce a slime layer was evaluated using the microtiter plate test (MtP) and Congo red tube test, respectively. Using PCR, the presence of the intercellular adhesion (*ica*) locus in *S. aureus* strains was confirmed. Subsequently, quantitative real-time RT-PCR was performed to investigate *icaA* transcription in various media, including tryptic soy broth (TSB), brain-heart infusion broth (BHIB), nutrient broth (NB), and Mueller-Hinton broth (MHB), containing 0, 0.25, 0.5, 1, and 2% glucose.

Results: Our results showed that although all studied strains adhered to the wells of polystyrene microtiter plates, the optimal rate of biofilm formation was observed in TSB medium containing 1% glucose. However, biofilm formation was not significantly different in NB, MHB, and BHIB media. Supplementation of all media with 1% glucose resulted in the highest biofilm production, and *icaA* transcription increased in all media after glucose addition up to 1%.

Conclusion: The results of the present study indicated that TSB medium supplemented with 1% glucose was the most appropriate medium for evaluating biofilm formation by *S. aureus* isolates.

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Introduction

Biofilms are adherent bacterial communities that irreversibly attach to surfaces 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, resulting in significant morbidity and mortality. Structural analysis of biofilms often reveals that bacterial biofilms are surrounded by an extracellular polymeric substance (EPS) (1,2). The gene expression pattern of bacteria in a biofilm differs from that of planktonic cells. This difference in gene expression, together with other characteristics, including the physical barrier formed by exopolysaccharides, can increase biofilm tolerance to antibiotics, disinfectants, and host immune mechanisms (3). *S. aureus* is one of the most important bacterial species capable of forming biofilms on biotic and abiotic surfaces (4-7). For instance, *S. aureus* biofilms may be involved in infections of central venous catheters, urinary catheters, prosthetic heart valves, orthopedic implants, and dialysis catheters. The EPS of *S. aureus* is mainly composed of poly-N-acetylglucosamine polymer, which is encoded by the *icaADBC* operon (6,8,9).

Given that *icaA*, a component of the *icaADBC* operon, encodes an essential enzyme in the biosynthesis of polysaccharide intercellular adhesin (PIA)-a critical factor in biofilm matrix formation-monitoring its transcription provides a molecular indicator of biofilm-forming potential under various culture conditions. Therefore, assessing *icaA* expression alongside phenotypic biofilm quantification helps define the optimal conditions for robust biofilm development in *S. aureus* (10,11).

Several phenotypic and genotypic methods are available for the detection of staphylococcal biofilms. Although widely used, phenotypic methods such as the MtP assay and Congo red agar (CRA) test may have limitations, including subjective interpretation, operator variability, and sensitivity to environmental conditions. These limitations highlight the need to complement them with molecular techniques, such as the quantification of *icaA* transcription, to ensure a more accurate evaluation of biofilm-forming capacity (12). Genotypic methods, such as PCR, determine the presence of biofilm-related genes, including those encoding microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (13,14). The MtP test uses 96-well microtiter plates to measure the optical density (OD) values of stained bacterial biofilms. This method provides quantitative results (14).

In the CRA method, bacteria are cultured on Congo red agar and incubated; thereafter, the colony color indicates their ability to produce a slime layer. Slime-forming strains appear as black colonies, whereas non-slime-forming strains appear red (15). In addition, MSCRAMMs mediate *S. aureus* attachment to different surfaces (16). Intercellular signaling between bacteria, also known as quorum sensing, has been shown to be involved in biofilm development by *S. aureus*. Quorum sensing occurs through 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 the accessory gene regulator (agr) locus. Activation of the agr system can affect *S. aureus* biofilm formation through the downregulation of MSCRAMMs as well as the production of a protease (17). It has been reported that bacterial species, attachment surface, and surrounding medium are three important factors involved in biofilm formation (18). The ability of each strain to adhere and form biofilm varies markedly across different culture media and environmental conditions (18). 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 (19,20). In this regard, one study showed that glucose may repress the agr system by lowering pH, resulting in increased biofilm formation (21). Thus, the present study was conducted to identify an appropriate bacterial culture medium with an optimal glucose concentration for examining the biofilm-forming ability of *S. aureus* strains.

Methods

Bacterial strains, culture media, and growth conditions

S. aureus ATCC 25923 was used as a reference biofilm-producing strain in all experiments. In addition, two *S. aureus* strains isolated from tracheal tube samples of patients admitted to the ICU of a hospital in Hamedan (western Iran) were included in the present study. These bacterial isolates were identified based on culture characteristics and biochemical tests, and their identity was further confirmed by a species-specific PCR assay targeting the *femA* gene of *S. aureus* (22). Meanwhile, *S. epidermidis* ATCC 12228 was used as a non-biofilm-producing 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 biofilm-forming ability using a previously described microtiter plate assay (23). Briefly, 50 µl of an overnight culture of each isolate was added to 96-well microplates (SPL, Korea) containing 50 µl of nutrient broth (NB), tryptic soy broth (TSB), Mueller-Hinton broth (MHB), or brain-heart infusion broth (BHIB) 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 groups. The microplates were aerobically incubated at 37 °C for 24 hours. The supernatants were then discarded, and the microplates were washed twice with phosphate-buffered saline (PBS). The plates were left to dry overnight and stained with 200 µl of 0.1% Safranin dye for 15 min, followed by three washes with 300 µl of PBS and overnight air-drying. Afterwards, 100 µl of ethanol-acetic acid (95:5 V/V) was added to each well to dissolve bound Safranin in adherent bacterial cells. Finally, optical density values were measured at 490 nm using a plate reader. The experiment was performed in triplicate. The biofilm-producing *S. aureus* ATCC 25923 and the non-biofilm-producing *S. epidermidis* ATCC 12228 were used as control strains (24).

Slime assay

The Congo red tube test was performed to evaluate slime production in different media supplemented with various glucose concentrations. For this purpose, a single colony from an overnight culture was inoculated into 10 ml of NB, TSB, MHB, or BHIB containing 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 groups. 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 color: black, weak black, and red. As exopolysaccharides directly react with Congo red and produce a black color, the intensity of black coloration was considered to reflect the amount of exopolysaccharides or polysaccharide intercellular adhesins (PIA) (25). Therefore, black and red media were considered to correspond to normal slime-producing and non-slime-producing isolates, respectively.

RNA extraction and cDNA synthesis

To evaluate *icaA* gene transcription, the presence of its encoding DNA was first investigated in *S. aureus* isolates and the reference strain using a PCR assay with previously introduced primers (26). The sequence of the forward primer was 5′-TATTCAATTTACAGTCGCAC-3′, and that of the reverse primer was 5′-GATTCTCTCCCTCTCTGCCA-3′. These primers amplify a 407 bp DNA fragment of *icaAD* based on a published sequence (AF086783). A PCR product was sequenced to confirm PCR specificity. To remove 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 absorbance at 260 nm using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Extracted RNAs were stored at -70°C for subsequent experiments. The purified RNA was then converted to cDNA according to the manufacturer's instructions (cDNA Synthesis Kit, Takara, Japan) and stored at -20°C for use as a template in real-time RT-PCR.

Relative quantitative real-time RT-PCR

SYBR Green real-time PCR Master Mix (Ampliqon, 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 the *16S rRNA* housekeeping gene was used as an internal control to normalize *icaA* expression levels. Amplification was performed as follows: denaturation at 95 °C for 10 min, followed by 40 cycles consisting of denaturation at 95 °C for 30 sec, annealing at 53 °C (For *icaA*) and 59 °C (For *16S rRNA*) for 30 sec, and extension at 72 °C for 30 sec. A negative control was included in each run. All samples were analyzed in triplicate, and relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (27).

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

Genes	Sequence (5′-3′)	Annealing temperature	Reference
icaA	GGAAGTTCTGATAATACTGCTG	53 °C	(28)
	GATGCTTGTTTGATTCCCTC		
16S rRNA	AGCCGACCTGAGAGGGTGA	59 °C	(29)
	TCTGGACCGTGTCTCAGTTCC		

Statistical analysis

One-way ANOVA was performed to compare OD values obtained from three independent experiments using SPSS software (Version 26). The mean difference was considered significant at $P < 0.05$.

Results

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

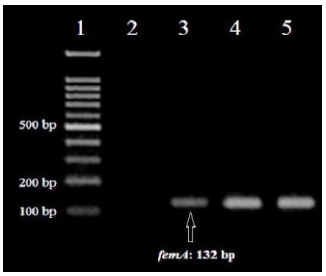

Figure 1. Agarose gel electrophoresis of species-specific PCR. Lane 1: 100 bp DNA ladder; Lane 2: Negative control (No template DNA); Lane 3: Positive control for the *femA* gene (*S. aureus* ATCC 25923); Lanes 4 and 5: PCR amplicons obtained from the isolates.

Microtiter plate assay

Biofilm-forming ability was investigated by measuring the OD values of the examined isolates using the MtP assay. In all tested media, the biofilm formation rate of *S. epidermidis* 12228 was significantly lower than that of *S. aureus* 25923 and the other two isolates. However, the results showed no significant difference in biofilm formation between *S. aureus* 25923 and the other isolates. Moreover, the highest and lowest rates of biofilm formation occurred when bacteria were cultured in TSB and NB, respectively. The biofilm formation patterns of the two clinical isolates were similar to those observed for *S. aureus* 25923 (Data are not shown). Addition of glucose from 0 to 1% in all media also had a significant positive effect on biofilm formation by *S. aureus* ($P < 0.05$), and the highest biofilm formation was observed in media containing 1% glucose. However, increasing the glucose concentration to 2% did not further promote biofilm formation.

Slime assay

In the Congo red tube test, *S. aureus* 25923 and the other two clinical isolates (Biofilm-producing 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 basal red color of any of the examined media. In other words, there was no significant difference in slime production among the different media as determined by the CRA method, as confirmed by unpaired Student's t-test ($P > 0.05$ for all pairwise comparisons) (Figure 2).

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

The results of 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 the PCR assay. The sequence obtained for the *icaA* amplicon was 99% identical to the corresponding GenBank sequence (Accession number WP_031785277.1). The results of real-time PCR indicated that *icaA* expression increased with the addition of glucose up to 1% in TSB, MHB, NB, and BHIB media. Figure 3 shows the results of real-time PCR for *icaA* expression.

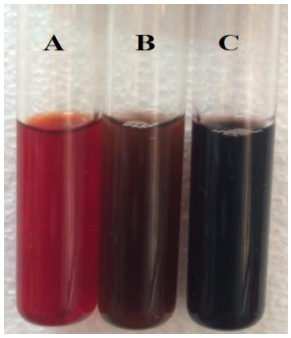

Figure 2. Screening of slime (PIA) producers by tube test. A: Negative slime producer (*S. epidermidis* ATCC 12228, red pigments); B: Moderate slime producer; and C: Positive slime producer (*S. aureus* ATCC 25923, black pigments).

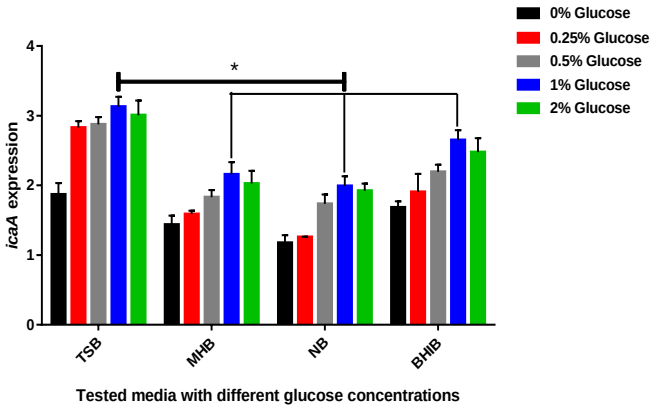

Figure 3. The *icaA* expression in various media containing 0, 0.25, 0.5, 1, and 2% glucose. The maximum *icaA* expression occurred in TSB supplemented with 1% glucose. Error bars represent the standard deviation of three replicates (*: P<0.05).

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 (30). In particular, the presence of glucose in the growth medium has been reported to enhance biofilm formation (31). Various methods have been introduced to measure the biofilm-forming capability of *S. aureus* isolates, among which the MtP assay is one of the most common methods used in bacteriology laboratories. 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 (23).

In the present study, the biofilm-forming ability of *S. aureus* strains was investigated in four bacterial culture media (NB, TSB, MHB, and BHIB) containing various glucose concentrations (0, 0.25, 0.5, 1, and 2%) using the MtP assay. The results showed that, among all tested broth media, TSB was the most suitable medium for examining biofilm-forming ability. However, some studies have reported that bacterial biofilm formation in BHIB can sometimes occur more effectively than in TSB (31-33). Nevertheless, it has also been shown that some staphylococcal strains produce greater biofilm in TSB than in BHIB (34). Moreover, researchers commonly use TSB with various glucose supplements for evaluating biofilm formation by *S. aureus* strains (33). It has been reported that the addition of glucose to culture media increases the biofilm-forming ability of staphylococci (32-34). In agreement with previous findings, the present study showed a significant increase in *S. aureus* biofilm production when the bacterium was exposed to increasing glucose concentrations. The optimal glucose concentration for biofilm formation of *S. aureus* on polystyrene surfaces in all tested culture media was 1%.

In addition, the 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 media with different glucose concentrations; however, the highest expression occurred when 1% glucose was added to the medium (P<0.05). Within the *icaADBC* operon, the *icaA* gene encodes the enzyme N-acetylglucosaminyl transferase, which catalyzes the synthesis of poly-N-acetylglucosamine polymer, known as PIA, and is important for cell-to-cell adhesion and accumulation (35). The *icaADBC* operon and products of the *ica* locus [*icaR* (Regulatory) and *icaADBC* (Biosynthetic) genes] have been demonstrated to be necessary for biofilm formation (36). Consequently, in the present study, the expression of the *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 highest *icaA* expression level was detected with 1% glucose supplementation. In addition, the color of the Congo red tube culture was not altered by glucose addition, indicating that this medium is not suitable for precise quantification of PIA or measurement of *icaADBC* operon expression.

The CRA assay did not reflect increased *icaA* transcription or biofilm formation in response to glucose supplementation, suggesting that this phenotypic method lacks the sensitivity required to detect metabolic or transcriptional modulation of biofilm-related genes, such as those in the *icaADBC* operon. Additionally, CRA relies on visual detection of slime production, which may not accurately correspond to PIA levels or gene expression, thereby limiting its utility in mechanistic studies (14).

The results of the present study revealed that glucose increases biofilm-forming ability by promoting *icaA* transcription and, subsequently, PIA production. Nevertheless, additional *ica*-independent factors may be involved in increasing phenotypic biofilm formation by staphylococci. For instance, it has been reported that the agr system is downregulated by glucose addition (21), and *S. aureus* agr mutants also show enhanced biofilm formation (37). Therefore, glucose-induced promotion of biofilm formation may be related to a suppression mechanism involving agr. Using phenotypic analysis of wild-type and mutant strains of *S. aureus*, Lim et al. also described that the rbf gene is another gene that mediates *S. aureus* biofilm formation at the multicellular aggregation stage in an *ica*-independent manner (38), suggesting that the genetic basis of biofilm formation in staphylococci is multifactorial and remains to be further explored.

Conclusion

The results of the present study suggest that, among the various media examined, TSB supplemented with 1% glucose was the most appropriate medium for evaluating biofilm formation by *S. aureus* strains. However, further studies are necessary to elucidate the precise mechanisms underlying the increase in *S. aureus* biofilm formation following glucose supplementation of culture media.

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Ethical Statement

All experiments and methods were performed in accordance with relevant guidelines and regulations. All experimental protocols were approved by a named institutional/licensing committee. Tracheal tube sample 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.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Author Contributions

ASH 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. All authors read and approved the final manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Use of Artificial Intelligence

The authors confirm that no artificial intelligence (AI) tools or AI-assisted technologies were used in the conception, writing, editing, data analysis, interpretation, or preparation of this manuscript. All aspects of the work were performed solely by the authors.

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