

Optimum cultural conditions to achieve the best biofilm formation and high level *icaA* transcription by *Staphylococcus aureus*

Running title: Biofilm formation and high level *icaA* transcription by *Staphylococcus aureus*

Aram Sharifi

Department of Animal Science, Faculty of Agriculture, University of Kurdistan, Sanandaj, Kurdistan, Iran

Email: a.sharifi@uok.ac.ir ORCID: 0000-0002-1416-9726

Abdolmajid Mohammadzadeh

Department of Pathobiology, Faculty of Veterinary Science, Bu-Ali Sina University, Hamedan, Iran

Email: Mohammadzadeh@basu.ac.ir ORCID: 0000-0001-8658-8164

Pezhman Mahmoodi

Department of Pathobiology, Faculty of Veterinary Science, Bu-Ali Sina University, Hamedan, Iran

Email: mahmoodi_pezhman@yahoo.com ORCID: 0000-0001-7150-6891

Taghi Zahraei Salehi

Department of Microbiology and Immunology, Faculty of Veterinary Science, Tehran University, Tehran, Iran

Email: tsalehi@ut.ac.ir ORCID: 0000-0002-5665-5757

Corresponding author: Abdolmajid Mohammadzadeh

Tel: +989189814502

Email: Mohammadzadeh@basu.ac.ir

Address: Department of Pathobiology, Faculty of Veterinary Science, Bu-Ali Sina University, Hamedan, Iran

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 to produce slime layer were evaluated using microtiter plate test (MtP) and Congo red tube test, respectively. Using PCR, the presence of intercellular adhesion (*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.

Results: 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 media transcription of *icaA* was increased with glucose addition to one present.

Conclusions: 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 words: Biofilm formation, *Staphylococcus aureus*, Culture media, Glucose, Quantitative real-time RT-PCR

Introduction

Biofilms are an adherent community of bacteria 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 and resulting in significant morbidity and mortality. Structural analysis of biofilms often reveals that bacterial biofilms are often surrounded by an extracellular polymeric substance (EPS) (1,2). 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 (3). *S. aureus* is one of the most important bacterial species which can form 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. *S. aureus* EPS is mainly composed of poly-N-acetylglucosamine polymer which is encoded by *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 cultural conditions. Therefore, assessing *icaA* expression alongside phenotypic biofilm quantification helps define 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 MtP assay and Congo red agar (CRA) test may suffer from limitations including subjective interpretation, operator variability, and sensitivity to environmental conditions. These limitations highlight the need to complement them with molecular techniques, such as quantifying *icaA* transcription, to ensure more accurate evaluation of biofilm-forming capacity (12). Genotypic methods like PCR, determine biofilm related genes e.g. those encoded microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (13,14). MtP test uses 96-well microtiter plates to measure the optical density (OD) values of stained bacterial biofilms. This method gives a quantitative result (14).

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 (15). In addition, the MSCRAMMs mediate *S. aureus* attachment to different surfaces (16). 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 (17). It has been described that bacterial species, attachment surface and surrounding medium are three important factors involved in the formation of biofilms (18). The ability of each strain to adhere and form biofilm is very different in various 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 case, it has been shown in a study that glucose may repress *agr* system by lowering pH which results in increased biofilm formation (21). 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 by *S. aureus* strains.

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* (22). 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 (23). 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 (24).

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) (25). 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 (26). 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 (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 *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 $2^{-\Delta\Delta\text{CT}}$ method (27).

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	(28)
	GATGCTTGTTTGATTCCCTC		
<i>16S rRNA</i>	AGCCGACCTGAGAGGGTGA	59°C	(29)
	TCTGGACCGTGTCTCAGTTCC		

Statistical Analysis

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

Results

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

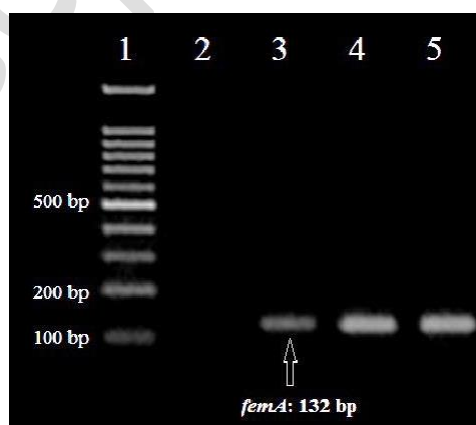


Figure 1. Agarose gel electrophoresis of species-specific PCR. Lane 1: 100 bp DNA ladder, lane 2: negative control (no template DNA), lane 3: positive control for *femA* gene (*S. aureus* ATCC 25923), lanes 4 and 5: PCR amplicons obtained from the isolates.

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.

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, 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).

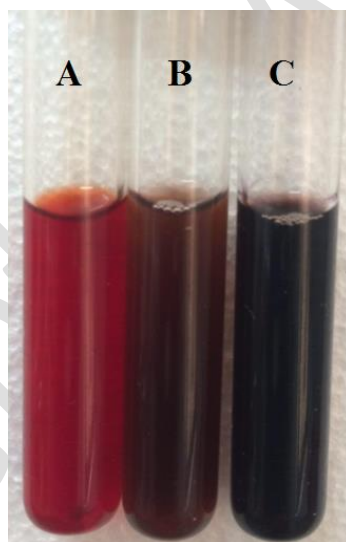


Figure 2. Screening of slime (PIA) producers by tube test. A: negative slime producer (*S. epidermidis* ATCC 12228, red pigments), B: moderate slime producer, and C: positive slime producer (*S. aureus* ATCC 25923, black pigments).

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 3 depicts the results of real time PCR for *icaA* expression.

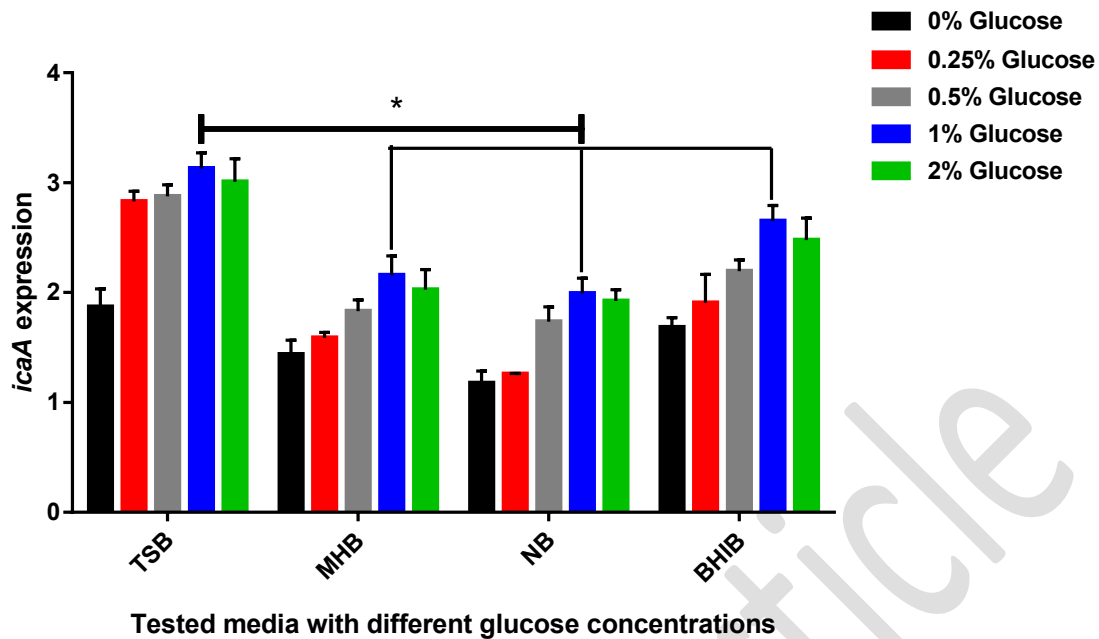


Figure 3. The *icaA* expression in various media contained 0, 0.25, 0.5, 1 and 2% glucose. The maximum *icaA* expression has occurred in TSB supplemented with 1% glucose. The error bars represent the standard deviation of three replicates (*: $P < 0.05$).

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, 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 (23).

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 (31-33). Nevertheless, it has also been shown that some staphylococcal strains produce greater biofilm in TSB compared to BHIB (34). Moreover, researchers commonly use TSB with various glucose supplements for evaluation of biofilm formation by *S. aureus* strains (33). It has been reported that addition of glucose to culture media lead to the increase of the ability of staphylococci biofilm formation (32-34). 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 (35). The *icaADBC* 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 *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 by glucose addition, indicating that this medium is not suitable for measurement of precise amount 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 sensitivity to detect metabolic or transcriptional modulation of biofilm-related genes such as those in the *icaADBC* operon. Additionally, CRA relies on the visual detection of slime production, which may not accurately correspond to PIA levels or gene expression, limiting its utility in mechanistic studies (14).

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 (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 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 (38), suggesting that the genetic basis of biofilm formation in staphylococci is multifactorial, and much remains to be explored.

Conclusion

the results of 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.

Acknowledgments

The authors would like to thank Bu-Ali Sina University.

Funding Sources

This research was supported by research grants (Grant NO: 95-200) from Bu-Ali Sina University of Hamedan, Iran.

Ethical Statement

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.

Conflict of Interest

The authors declare that they have no conflict of interests.

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.

References

1. Kim J-H, Han S-Y, Kwon J-H, Lee D-S. *Orostachys japonicus* ethyl acetate fraction suppresses MRSA biofilm formation. *Asian Pac J Trop Med*. 2020;13:38.
2. Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME. *Staphylococcus aureus* biofilms: properties, regulation, and roles in human disease. *Virulence*. 2011;2:445-59.
3. Lewis K. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol*. 2007;5:48-56.
4. Yu D, Zhao L, Xue T, Sun B. *Staphylococcus aureus* autoinducer-2 quorum sensing decreases biofilm formation in an *icaR*-dependent manner. *BMC Microbiol*. 2012;12:1-12.
5. Sharifi A, Mohammadzadeh A, Zahraei Salehi T, Mahmoodi P, Nourian A. *Cuminum Cyminum* L. essential oil (CcEO): A promising antibacterial and anti-virulence agent against multidrug-resistant *Staphylococcus aureus*. *Front Microbiol*. 2021;12:2016.
6. Sharifi A, Mohammadzadeh A, Zahraei Salehi T, Mahmoodi P. Antibacterial, antibiofilm and anti-quorum sensing effects of *Thymus daenensis* and *Satureja hortensis* essential oils against *Staphylococcus aureus* isolates. *J Appl Microbiol*. 2018;124:379-88.
7. Sharifi A, Sobhani K, Mahmoudi P. A systematic review and meta-analysis revealed a high-level antibiotic resistance of bovine mastitis *Staphylococcus aureus* in Iran. *Res Vet Sci*. 2023.
8. Francois P, Schrenzel J, Stoerman-Chopard C, Favre H, Herrmann M, Foster TJ, et al. Identification of plasma proteins adsorbed on hemodialysis tubing that promote *Staphylococcus aureus* adhesion. *J Lab Clin Med*. 2000;135:32-42.
9. Sharifi A, Mahmoudi P, Sobhani K. The prevalence of adhesion and biofilm genes in *Staphylococcus aureus* isolates from bovine mastitis: A comprehensive meta-analysis. *Vet Med Sci*. 2024;10:e31378.
10. Rahmanian N, Moulavi P, Ashrafi F, Sharifi A, Asadi S. Surface-functionalized UIO-66-NH₂ for dual-drug delivery of vancomycin and amikacin against vancomycin-resistant *Staphylococcus aureus*. *BMC Microbiol*. 2024;24(1):462.
11. Arciola CR, Campoccia D, Ravaioli S, Montanaro L. Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. *Front Cell Infect Microbiol*. 2015;5:7.
12. Christensen GD, Simpson WA, Younger J, Baddour L, Barrett F, Melton D, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a

- quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol.* 1985;22:996-1006.
13. Foster TJ. The MSCRAMM family of cell-wall-anchored surface proteins of gram-positive cocci. *Trends Microbiol.* 2019;27(11):927-41.
 14. Sharifi A, Mahmoudi P, Sobhani K, Ashengroph M. The Prevalence and Comparative Analysis of Adhesion and Biofilm-Related Genes in *Staphylococcus aureus* Isolates: A Network Meta-Analysis. *Microbiol Immunol.* 2025; 69(2):104-13.
 15. Freeman D, Falkiner F, Keane C. New method for detecting slime production by coagulase negative staphylococci. *J Clin Pathol.* 1989;42:872-4.
 16. Seo Y-S, Lee DY, Rayamahji N, Kang ML, Yoo HS. Biofilm-forming associated genotypic and phenotypic characteristics of *Staphylococcus* spp. isolated from animals and air. *Res Vet Sci.* 2008;85:433-8.
 17. Yarwood JM, Bartels DJ, Volper EM, Greenberg EP. Quorum sensing in *Staphylococcus aureus* biofilms. *J Bacteriol.* 2004;186:1838-50.
 18. Van Houdt R, Michiels C. Biofilm formation and the food industry, a focus on the bacterial outer surface. *J Appl Microbiol.* 2010;109:1117-31.
 19. Di Bonaventura G, Stepanović S, Picciani C, Pompilio A, Piccolomini R. Effect of environmental factors on biofilm formation by clinical *Stenotrophomonas maltophilia* isolates. *Folia Microbiol.* 2007;52:86-90.
 20. Kalia VC, Prakash J, Koul S, Ray S. Simple and rapid method for detecting biofilm forming bacteria. *Indian J Microbiol.* 2017;57:109-11.
 21. Regassa LB, Novick RP, Betley MJ. Glucose and nonmaintained pH decrease expression of the accessory gene regulator (*agr*) in *Staphylococcus aureus*. *Infect Immun.* 1992;60:3381-8.
 22. Kobayashi N, Wu H, Kojima K, Taniguchi K, Urasawa S, Uehara N, et al. Detection of *mecA*, *femA*, and *femB* genes in clinical strains of staphylococci using polymerase chain reaction. *Epidemiol Infect.* 1994;113:259-66.
 23. Dhanawade NB, Kalorey DR, Srinivasan R, Barbuddhe SB, Kurkure NV. Detection of intercellular adhesion genes and biofilm production in *Staphylococcus aureus* isolated from bovine subclinical mastitis. *Vet Res Commun.* 2010;34:81-9.
 24. He J-z, Wang A-q, Liu G, Gao J, Ali T, Han B. Biofilm Formation and Biofilm-Associated Genes Assay of *Staphylococcus aureus* Isolated from Bovine Subclinical Mastitis in China. *Pak Vet J.* 2014;34.
 25. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect Immun.* 1982;37:318-26.
 26. Yazdani R, Oshaghi M, Havaei A, Pishva E, Salehi R, Sadeghizadeh M, et al. Detection of *icaAD* gene and biofilm formation in *Staphylococcus aureus* isolates from wound infections. *Iran J Public Health.* 2006; 35(2): 25-28.
 27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods.* 2001;25:402-8.
 28. Harapanahalli AK, Chen Y, Li J, Busscher HJ, van der Mei HC. Influence of adhesion force on *icaA* and *cidA* gene expression and production of matrix components in *Staphylococcus aureus* biofilms. *Appl Environ Microbiol.* 2015;81:3369-78.
 29. Koprivnjak T, Mlakar V, Swanson L, Fournier B, Peschel A, Weiss JP. Cation-induced transcriptional regulation of the *dlt* operon of *Staphylococcus aureus*. *J Bacteriol.* 2006;188:3622-30.
 30. Rohde H, Knobloch JK-M, Horstkotte MA, Mack D. Correlation of biofilm expression types of *Staphylococcus epidermidis* with polysaccharide intercellular

- adhesin synthesis: evidence for involvement of *icaADBC* genotype-independent factors. *Med Microbiol Immunol.* 2001;190:105-12.
31. Stepanović S, Vuković D, Hola V, BONAVENTURA GD, Djukić S, Ćirković I, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Apmis* 2007;115:891-9.
 32. Mathur T, Singhal S, Khan S, Upadhyay D, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. *Indian J Med Microbiol.* 2006;24:25-9.
 33. Kennedy CA, O'Gara JP. Contribution of culture media and chemical properties of polystyrene tissue culture plates to biofilm development by *Staphylococcus aureus*. *J Med Microbiol.* 2004;53:1171-3.
 34. Knobloch JK-M, Horstkotte MA, Rohde H, Mack D. Evaluation of different detection methods of biofilm formation in *Staphylococcus aureus*. *Med Microbiol Immun.* 2002;191:101-6.
 35. Gerke C, Kraft A, Süßmuth R, Schweitzer O, Götz F. Characterization of the N-Acetylglucosaminyltransferase Activity Involved in the Biosynthesis of the *Staphylococcus epidermidis* Polysaccharide Intercellular Adhesin. *Journal of Biol Chem.* 1998;273:18586-93.
 36. Croes S, Deurenberg RH, Boumans M-LL, Beisser PS, Neef C, Stobberingh EE. *Staphylococcus aureus* biofilm formation at the physiologic glucose concentration depends on the *S. aureus* lineage. *BMC Microbiol.* 2009;9:1-9.
 37. Vuong C, Saenz HL, Götz F, Otto M. Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J Infect Dis.* 2000;182:1688-93.
 38. Lim Y, Jana M, Luong TT, Lee CY. Control of glucose- and NaCl-induced biofilm formation by rbf in *Staphylococcus aureus*. *J Bacteriol.* 2004;186:722-9.