

Association of *agr* Gene Expression with *Staphylococcus aureus* Virulence Genes in BHI Broth

Hadi Koohsari (PhD)

Department of Microbiology, Azadshahr Branch, Islamic Azad University, Azadshahr, Iran

Ezzat Allah Ghaemi (PhD)

Department of Microbiology, Golestan University of Medical Sciences, Gorgan, Iran

Nour Amir Mozaffari (PhD)

Department of Microbiology, Iran University of Medical Sciences, Tehran, Iran

Abdolvahab Moradi (PhD)

Department of Microbiology, Golestan University of Medical Sciences, Gorgan, Iran

Corresponding Author: Hadi Koohsari

E-mail: hadikoohsari@yahoo.com

Tel: +989112730361

Address: Department of Microbiology, Azadshahr Branch, Islamic Azad University, Azadshahr, Iran

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ABSTRACT

Background and Objective: Agr is the most important regulatory system for the expression of *Staphylococcus aureus* virulence factors in different conditions. Agr acts as a quorum sensing system in this bacterium which is activated by increased cell concentration during the transition from exponential growth phase to stationary phase. Its role is to upregulate the secretory virulence factors such as alpha-hemolysin and inhibit the transcription of surface proteins including protein A-encoding gene. The aim of this study was to assess the relationship between the agr system expression and some virulence factors of *Staphylococcus aureus* in Brain-heart infusion (BHI) broth.

Methods: The expression level of *agrA* and RNAPIII genes from the *agr* locus along with the expression of *hla*, *spa* and *mecA* genes in BHI broth were assessed in different growth phases using Real time-PCR. Also, *gyrB* was used as an internal control in this study.

Results: The growth curve of the five tested isolates in BHI broth at 24 hours showed that all the isolates had relatively similar growth patterns. AgrA gene expression in the stationary phase was decreased by 0.89-fold compared with the exponential phase. Although the expression of RNAPIII gene increased by 3-fold, *hla* expression decreased by 0.47-fold.

Conclusion: An inactive *agr* system is observed in the BHI broth medium. BHI broth medium contains high amounts of suitable nutrients for the growth of *Staphylococcus aureus*, thus the bacteria do not require the activity of the *agr* system for the regulation of the virulence genes in these conditions.

Keywords: *Staphylococcus aureus*; *agr*; Virulence Factors; Growth Curve; BHI Broth.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is an opportunistic pathogen and one of the most important health problems in the world. It is a commensal microflora of almost 30% of the adult population (1). This bacterium is associated with a variety of human diseases ranging from benign skin infections to life-threatening systemic diseases such as osteomyelitis, endocarditis, pneumonia, bacteremia, sepsis and toxic shock syndrome (2). The increasing emergence of Methicillin-resistant *S. aureus* (MRSA) strains, which can cause problems such as nosocomial infections, has increased the importance of these bacteria (3). The ability of these bacteria to cause a wide range of diseases is related to expression of a large number of virulence factors. The most important system that regulates the expression of virulence factors in *S. aureus* is known as accessory gene regulator (*agr*), which is also the first known regulatory system in this bacterium (4). The *agr* is the intermediate of regulating more than 70 genes, 24 of which have been identified as virulence factors (4, 5). It is considered as a quorum sensing system in *S. aureus* that is activated following the increase of intracellular concentration during the transition from exponential phase to stationary phase of growth. It plays a regulatory role by increasing the transcription of genes involved in exoprotein production and toxin secretion such as the gene encoding alpha-hemolysin (*hla*), inhibiting the transcription of genes involved in binding of bacteria to host cells and evading the immune system such as protein a encoding gene (*spa*) (4,6). The *agr* locus consists of two operons, P2 and P3 which express two different transcripts. The P2 operon expresses RNAII transcript which contains four genes (*agrA*, *agrB*, *agrC* and *agrD*) that encode four proteins. The activity of AgrB which is a membrane-bound protein, causes the secretion of AgrD, also known as the autoinducing peptide (AIP). Once sufficient quantities of the *agrD* (AIP) accumulates in the culture medium (usually during the transition from the exponential phase to the stationary phase), AgrC (a membrane-bound extracellular kinase that binds to the extracellular AIP) becomes activated. Following this activation, AgrC activates the main regulator (AgrA) through its phosphorylation. AgrA also operates to increase the transcription of RNAII and

RNAIII. P3 operon expresses RNA III transcript which acts as an effector molecule of the *agr* locus. RNAIII is a 510-nucleotide molecule that stimulates the transcription of genes encoding secretory toxins and enzymes, while inhibiting the transcription of surface protein-encoding genes. In addition to its regulatory role, RNA III acts as an mRNA for staphylococcal delta-hemolysin (7,8).

This study aimed to investigate the gene expression level of *agrA* and RNAIII from the *agr* locus as well as the expression of *hla*, *spa* and *mecA* genes in five *S. aureus* isolates in BHI broth culture medium, during various growth phases. *gyrB* was also used as an internal control in this study.

MATERIAL AND METHODS

In this study, four *S. aureus* isolates were used, two of which were isolated from the nasal cavity of healthy volunteers (one MRSA and one MSSA) and the other two from patients (one MRSA and one MSSA). A standard *S. aureus* strain (COL) was also used in addition to these strains. The isolates were recovered and then was identified using conventional laboratory techniques including the Gram staining, catalase test, hemolysis on Blood agar and coagulase test. All the isolates were cultured on Blood agar for 24 hours at 37 °C. Also, 2-3 identical colonies from each isolate were inoculated into BHI broth media. The samples were kept at 37 °C until their suspension turbidity reached 0.5McFarland standard (1.5×10^8 CFU/ml).

The final suspension containing 10^3 CFU/ml was prepared by repeated dilution. This concentration was considered as 0-time, inoculation time. These cultures incubated at 37°C in 24 test tubes which were sampled at hourly interval. Surface culture on Mueller Hinton agar medium was done and then the samples were incubated at 37 °C.

The number of colonies were counted after 24-hour incubation. The total number of bacteria per hour was calculated by the following equation: The number counted colony \times 1/dilution index and the growth curve was plotted. After plotting the growth curves, bacteria from the exponential and stationary growth phases were used for RNA extraction and Real-time PCR. RNA extraction and purification RNA extraction was performed using the RNX-plus solution (CinnaGen Co.) according to the manufacturer's protocol. The

quality and integrity of the obtained RNA was assessed using agarose gel electrophoresis and ethidium bromide staining to detect two 1.5kb (16S) and 2.9 (23S) kb bands. In order to remove the residual amounts of DNA, RNase-free DNase I (Thermo Scientific) was used according to the Fermentas protocol. After choosing appropriate primers, Real-time PCR was performed using SYBR Green dye to assess the extracted level of the aforementioned RNA genes. The One-Step RT-PCR method was done using QuantiFast SYBR Green kit (Qiagen). RT-PCR was performed using an ABI Prism 7300 thermocycler (Applied Biosystems) and the level of targeted genes' expression in the extracted RNA samples from different isolates were normalized based on the *gyrB* gene as an internal control. Table 1 shows the list of used primers in this study which were obtained based on pairing location and length of the PCR product in NCBI website and using the Blast tool. The relative quantification method

was used in this study to assess gene expression. Expression of the genes were compared with a reference gene as an internal control (*gyrB*). Experiment was replicated three times. After performing the PCR process, The computer software constructs amplification plots using the fluorescence emission data that are collected during the PCR amplification. A fluorescence signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (C_T) for a sample. The amount of targeted gene in samples was normalized using a housekeeping gene as an internal control (ΔC_T). ΔC_T is the C_T of the target gene subtracted from the C_T of the housekeeping gene ($\Delta C_T = C_{T,target} - C_{T,gyrB}$). It should be noted that lower ΔC_T levels indicate higher gene expression levels.

RESULTS

The growth curve of the five *S.aureus* isolates in BHI broth during the incubation period from the inoculation time (0-time) up to 24 hours was plotted. Figure 1 demonstrates the growth curve of the five tested isolates in BHI broth in 24 hours which indicates that all of the isolates had relatively similar growth patterns (Figure 1).

Table 1- The list of primers used in this study

Name	Sequence (5' to 3')	Product size(bp)
<i>agrA</i> (F)	TGA TAA TCC TTA TGA GGT GCT T	163
<i>agrA</i> (R)	CAC TGT GAC TCG TAA CGA AAA	
<i>RNAlII</i> (F)	CGA TGT TGT TTA CGA TAG CTT	146
<i>RNAlII</i> (R)	CCA TCC CAA CTT AAT AAC CA	
<i>hla</i> (F)	GGG GAC CAT ATG ATA GAG ATT	154
<i>hla</i> (R)	TGT AGC GAA GTC TGG TGA AA	
<i>spa</i> (F)	GAT GGT AAC GGA GTA CAT GTC GTT	163
<i>spa</i> (R)	TTG CTG GTT GCT TCT TAT CAA CA	
<i>mecA</i> (F)	ACT GCT ATC CAC CCT CAA AC	165
<i>mecA</i> (R)	CTG GTG AAG TTG TAA TCT GG	
<i>gyrB</i> (F)	CGC AGG CGA TTT TAC CAT TA	141
<i>gyrB</i> (R)	GCT TTC GCT AGA TCA AAG TCG	

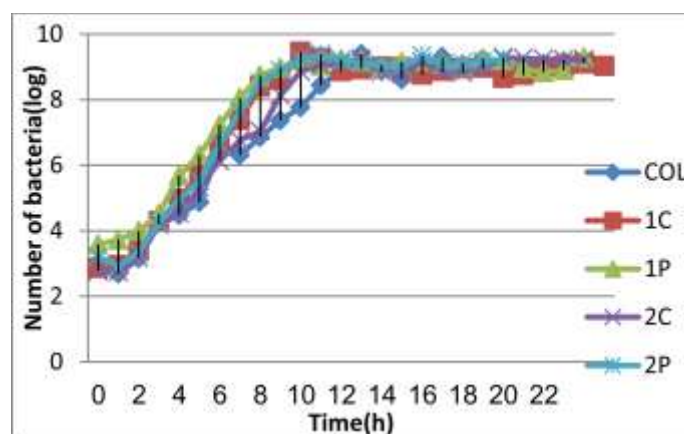


Figure 1- Growth curves of the five tested *S.aureus* isolates in BHI broth medium. All isolates had relatively similar growth patterns. Standard isolate (COL), clinical isolate MRSA (2P), clinical isolate of MSSA (1P), carrier isolate MRSA (2C), carrier isolate MSSA (1C).

The results showed that the doubling time of the standard strain (COL) was higher than the other strains. MRSA isolates showed higher doubling times compared to other MSSA isolates. Also, the mean doubling time of the clinical isolates was lower than the healthy carriers. The required time for division of the standard strain (COL), methicillin resistant clinical isolate (2P), methicillin-susceptible clinical isolate (1P), methicillin-resistant carrier isolate (2C) and methicillin-susceptible carrier isolate (1C) was 31.88, 24.52, 21.39, 27.07 and 22.07 minutes, respectively.

The device software showed the C_T of the studied genes in the five *S. aureus* isolates and then ΔC_T was calculated based on the *gyrB* gene (internal control) (Table 2).

Figure 3 shows the mean ΔC_T of different genes in the exponential and stationary phase. Since lower numerical amount of ΔC_T indicates increased gene expression, it can be concluded that gene expression of *agrA* and *hla* was decreased in the stationary phase and in contrast, *RNAIII*, *spa* and *mecA* genes showed higher expression levels in the

stationary phase (Figure 3).

The level of *agrA* gene expression in total of the five isolates showed a 0.89-fold increase during the transition from exponential phase to stationary phase. Whereas, the other regulatory gene of the *agr* system (*RNAIII*) showed a 3-fold increase in total of the five isolates in stationary phase compared to the exponential phase.

The gene expression of *hla* gene (alpha-hemolysin-encoding gene) showed a 0.47-fold decrease in the stationary phase. *Spa* gene (protein A-encoding gene) was evenly expressed in the exponential and stationary phase and had a 1.1-fold expression increase in the stationary phase. A 2.8-fold increase was observed in the case of *mecA* gene (methicillin-resistance encoding gene). *Spa* gene was expressed 2 times more in the clinical isolates compared with the carrier isolates, while *agrA* gene was 0.35-fold underexpressed. Also, the overexpression of *hla* and *RNAIII* genes in the clinical isolates was not significant when compared to the carrier isolates.

Table 2- ΔC_T of the five *S. aureus* isolates genes in the exponential and stationary phases in BHI broth

1C		1P		2P		2C		COL		
STA	EXP	STA	EXP	STA	EXP	STA	EXP	STA	EXP	
0.93	0.56	1.51	0.88	2.96	3.3	0.54	0.61	3.46	3.24	<i>agrA</i>
2.06	3.58	-1.12	1.99	2.49	4.8	1.47	1.68	6.42	7.15	<i>RNAIII</i>
1.52	0.75	1.62	-0.11	1.1	0.56	2.5	0.19	3.8	3.73	<i>Hla</i>
-0.73	0.15	-2	-0.34	-0.83	-0.44	1.03	0.18	5.79	4.44	<i>Spa</i>
MSSA	MSSA	MSSA	MSSA	0.96	1.85	8.8	11.75	-1.27	-0.67	<i>mecA</i>

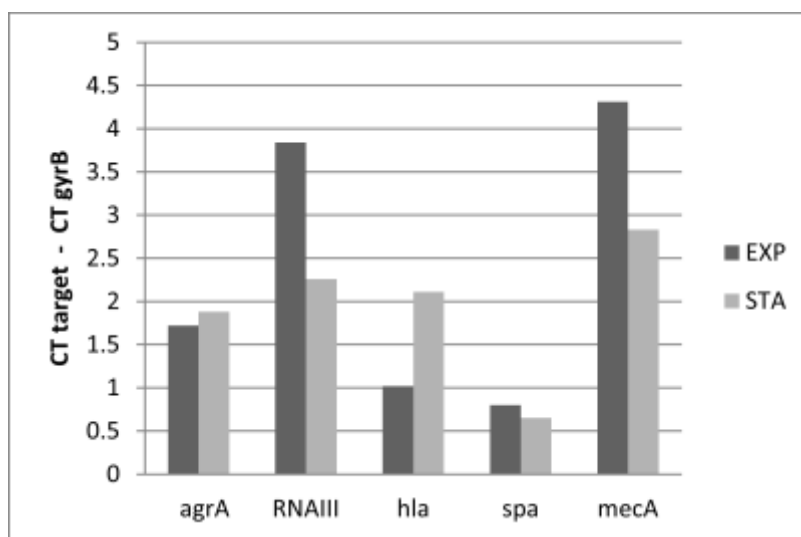


Figure 3 - Mean ΔC_T of different genes in the exponential and stationary phase in BHI broth

DISCUSSION

One of the aims of this study was to investigate the growth curve patterns of different *S. aureus* strains in BHI broth medium. As it was shown in Figure 1, the tested *S. aureus* isolates in this study had relatively similar growth patterns. Although the growth rate of COL standard strain was lower than other strains, eventually they all showed similar growth patterns. The findings of Laurent and Orozгани in two separate studies showed that MRSA isolates have longer doubling time compared with MSSA isolates which is in agreement with our findings (11, 10). MRSA strains contain the *mecA* gene that encodes PBP2A protein which is replaced on the bacterial surface and possibly prevent the entry of nutrients into the cells. If this theory is correct, the difficult entry of drugs and disinfectants into the MRSA isolates, ultimately making them more resistant to these compounds compared to the MSSA isolates is justified. Assessing the growth curve of the isolated *S. aureus* strains also showed that the isolated samples from healthy carriers had longer generation time compared to the clinical isolates. This is consistent with the results of Chen et al. which showed that the average doubling time for *S. aureus* isolates from nose of healthy subjects were higher compared to the clinical isolates (12). An inactive *agr* system is observed in BHI broth medium because despite our expectations, the regulator of the *agr* reaction (*agrA*) is less expressed during the transition from the exponential phase to the stationary phase. Even the expression of RNAIII was unable to exert regulatory effects on the *hla* gene as evidenced by the 0.47-fold decreased expression this gene in the stationary phase.

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As expected, the methicillin-resistance gene (*mecA*) was increased by 2.8 times compared to the exponential phase in these conditions. However, the *spa* gene expression change was not significant (Figure 4). These are in all agreement with Oogai et al. findings which showed that in Trypticase soy broth (TSB), RNAIII regulatory gene was overexpressed in the stationary phase, while accompanied by decreased expression of some virulence genes such as *aur* (aerolysin-encoding gene) and Panton-Valentine, with no change in their expression in the stationary phase in optimal conditions (13). The probable reason for this could be that in BHI broth, bacteria do not isolate, while *hlg* gene expression in the invasive isolates was higher than the colonized isolates. This group concluded that the origin of isolation cannot be involved in its pathogenicity without considering other genetic factors related to the isolate (18), which could explain the differences between our study and AbuOthman.

CONCLUSION

As mentioned previously, gene expression varies depending on the growth conditions. Therefore, assessing the gene expression in clinical conditions is recommended in order to obtain a more thorough understanding of virulent genes' expression and the effect of *agr* gene on patients and healthy carriers.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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